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# Phosphorylase of the Jack Bean: Its Purification, Estimation and Properties

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## INTRODUCTION

Cori and Green (1) found rabbit muscle phosphorylase to give a test for pentose. The prosthetic group-removing enzyme as well as trypsin (2) were shown to convert phosphorylase *a* into the inactive phosphorylase *b*. Ting (3) has shown that blood tryptase has this action also. The substance split from phosphorylase *a* by trypsin contains a pentose but this is not adenylic acid, although phosphorylase *b* is reactivated by adding adenylic acid. Upon dialyzing, this reactivated phosphorylase *b* activity is lost rapidly, since the adenylic acid is removed. Thus, it can be seen that considerable uncertainty exists regarding the prosthetic group of animal phosphorylase and its relation to adenylic acid.

Our knowledge of the properties and chemical nature of the vegetable phosphorylases is as yet even less complete than that of animal phosphorylases. Hanes (4) has purified pea phosphorylase and potato phosphorylase. Green and Stumpf (5) concentrated potato phosphorylase 370-fold.

In this laboratory we have been able to concentrate jack bean phosphorylase 900 to 1000-fold and find that the purified material contains about 4  $\mu$ g. of flavine adenine dinucleotide mg. All of this dinucleotide is firmly bound and cannot be removed by dialysis. However it is split off by heating. Our highly purified phosphorylase has no action upon Cori ester unless a primer is present. We find that the amylose of potato starch is an excellent primer, while the amylopectin is not. We find that under the conditions of our quantitative tests from 5 to 10 mg. of amylose is ample. Jack bean phosphorylase is rapidly destroyed by crystalline trypsin, but not by the prosthetic group-removing enzyme from rabbit muscle. Its pH of optimum

activity is 6.4. Its temperature optimum is at about 55°C'. It is activated neither by cysteine, nor by plant or animal adenylic acids. Unlike animal phosphorylase, it is not inhibited by D-glucose.

## EXPERIMENTAL

### *Purification of Phosphorylase*

Jack beans were ground to a fine powder in a micro-pulverizer.<sup>1</sup> The phosphorylase was extracted by mixing 100-g. lots of meal with 500-ml. portions of ice-cold 32% acetone and filtering overnight in an ice chest. The filtrate was centrifuged clear in a refrigerated centrifuge. The clear supernatant was chilled to - 5° to - 10°C', and to each 100 ml. of this was added, with rapid mixing, 70 ml. of acetone at - 5° to - 10°C. The precipitated phosphorylase was centrifuged down as rapidly as possible in a refrigerated centrifuge. The supernatant liquid was discarded and the precipitate was immediately dissolved in a little 0.5 *M* succinate buffer of pH 6.4 and dialyzed in the ice chest for 5-10 hr. against several changes of cold water.

The enzyme solution was next centrifuged clear and to every 100 ml. of supernatant was added 67 ml. of saturated ammonium sulfate neutralized with ammonia. After standing for several hours in the ice chest, the preparation was centrifuged in the refrigerated ice chest and the precipitate was discarded. The phosphorylase was now salted out by adding 50 ml. of neutral saturated ammonium sulfate to every 100 ml. The preparation was left in the ice chest for several hours and then centrifuged in 250-ml. bottles. As much as possible of the clear supernatant was carefully sucked off and the precipitate was transferred to 50 ml. centrifuge tubes and centrifuged down hard. The clear supernatant was sucked off and the sediment mixed with the smallest possible amount of succinate buffer pH 6.4 and pipetted into a dialyzing parchment. The dialysis was carried on in the ice chest for about 1 week. The precipitate was then centrifuged down and the supernatant poured off. Further purification was accomplished by fractional precipitation of the phosphorylase in the supernatant, using neutral ammonium sulfate.

Some phosphorylase is carried down during the dialysis mentioned above and this can be recovered by stirring the precipitate with succinate buffer and centrifuging clear.

<sup>1</sup> Bantam Micro-Pulverizer, Pulverizing Machinery Co., Summit, New Jersey.

On the whole this method for the purification of phosphorylase is not entirely satisfactory, since the yield is low. We have tried extracting jack bean meal with 30% alcohol and precipitating the enzyme with cold 60% alcohol. We have also extracted the phosphorylase from jack bean meal with 40% ammonium sulfate and have precipitated the enzyme in the extract by adding ammonium sulfate to 60% saturation. We have also precipitated the phosphorylase in 32% acetone extracts at neutrality with lead acetate and have then decomposed the lead complex with phosphate at pH 8.0. None of these procedures has been found especially satisfactory.

#### *Determination of Phosphorylase Activity*

Place 2 tubes graduated at 50 ml. in a thermostat-controlled water bath at 25°C. Add to each tube 1 ml. of properly diluted phosphorylase and 1 ml. of 1% starch amylose previously brought to 25°C. Add to one tube 1 ml. of 1% Cori ester in succinate buffer pH 6.4 and mix. At the end of 5 min. add to both tubes 5 ml. of 6.66% molybdate solution. This inactivates the enzyme. Now add 1 ml. of Cori ester to the tube to which none was added at the start. This is the blank test. Dilute both tubes to about 30 ml., add 5 ml. of 7.5 *N* sulfuric acid and mix. Add 5 ml. of freshly prepared 4% ferrous sulfate (6), dilute to the 50-ml. mark, stopper with a clean rubber stopper, mix and read in the photoelectric colorimeter, using a red filter. The readings are referred to a graph that has been prepared previously. The value of the blank is subtracted from the value of the test. This blank value should not amount to more than 0.01 mg. of phosphorus. Our phosphorylase unit is expressed in mg. of phosphorus set free from Cori ester at 25°C. at pH 6.4 in 5 min.

As shown in Fig. 1, a straight line is obtained when the phosphorylase units are plotted against the milliliters of phosphorylase employed, up to a value of 0.23 units. Therefore for greatest accuracy the test must be repeated, using a weaker enzyme solution, if values greater than 0.23 units are obtained.

#### *Preparation of Reagents*

Cori ester was synthesized according to the modification of Sumner and Somers (7) and was recrystallized three times. To prepare a solution free from inorganic phosphate, dissolve 1 g. of the dipotassium salt of Cori ester in 10 ml. of water. Add a pinch of calcium oxide, mix well and allow to stand for 5 or 10 min. Filter and wash the material through the filter with a little water. Add to the clear filtrate 2 or 3 drops of phenol red and then, drop by drop, enough normal sulfuric acid to bring to neutrality. Now dilute to 40 ml., add 60 ml. of succinate buffer, and mix. Keep this solution in the ice chest.

*Succinate Buffer.* Weigh 59 g. of c.p. succinic acid and 81 g. of c.p. sodium bi-

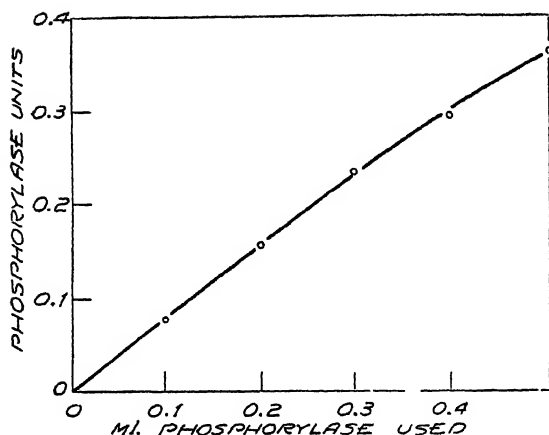


FIG. 1. Standardization curve for jack bean phosphorylase.

carbonate into a 1-l. beaker. Cautiously add distilled water and boil to remove carbon dioxide. Cool, dilute to 100 ml., and mix. The pH should be 6.4. This is not a very good buffer at this pH but we have not yet found one that is better.

*Starch Amylose.* Add 100 ml. of succinate buffer to 800 ml. of distilled water, heat to boiling, and turn off the flame. Add 10 g. of potato starch suspended in 100 ml of water and stir. Cool to room temperature and centrifuge clear. Decant the clear solution of amylose, preserve with toluene, and keep at room temperature. Do not place in the ice chest or reversion will occur.

### *Test for Flavine Adenine Dinucleotide*

Pipette into a small test tube 0.1–1.0 ml. of phosphorylase and heat in boiling water for 1 min. Cool and add 1 ml. of 1% DL-alanine in pyrophosphate buffer of pH 8.6. This contains 10 ml. of 8.7%  $\text{Na}_2\text{P}_2\text{O}_7 \cdot 7\text{H}_2\text{O}$ , 0.8 ml. of 1 *N* hydrochloric acid and 9.2 ml. of water (8); now add 1 ml. of solution containing 1 mg. of the apoenzyme of D-amino acid oxidase (9). Mix and allow the test to digest in a water bath at 25°C. for 30 min. At the end of this time add 1 ml. of half-saturated 2,4-dinitrophenylhydrazine in 1 *N* hydrochloric acid (10). Mix and allow to stand at room temperature for 15 min. Now add 3 ml. of 2 *N* sodium hydroxide. Dilute to 30 ml., mix, and read in the colorimeter, using a blue glass filter.

Standards are made by running tests in the same manner, using 2, 4, 6, 8, and 10  $\mu\text{g}$ . of pure flavine adenine dinucleotide and comparing the reading of the unknown with a graph made from these. It is also highly important to run a blank where the D-amino acid apoenzyme is not added until after the 2,4-dinitrophenylhydrazine has been added. This blank will show whether any acetone or other interfering substance

is present. The blank test should show practically no color. If it is colored the reagents will have to be purified, or else discarded.

#### ACKNOWLEDGMENT

We wish to thank the Rockefeller Foundation for financial support and Dr. P. S. Krishnan for preparing D-amino acid apoenzyme as well as flavine adenine dinucleotide.

#### SUMMARY

A method is described whereby jack bean phosphorylase can be concentrated 900 to 1000-fold. This purified material contains about  $\mu\text{g.}$  of bound flavine adenine dinucleotide/mg. The determination and properties of the enzyme are described.

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# The Competitive Effects of Metallic Ions on Citrate Oxidation <sup>1</sup>

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Received June 6, 1949

## INTRODUCTION

BaCl<sub>2</sub> and MgCl<sub>2</sub> inhibit citrate utilization by rabbit kidney cortex homogenates (1). MgCl<sub>2</sub> was more effective than BaCl<sub>2</sub> in this respect even though Mg citrate is soluble and Ba citrate insoluble. Therefore, insolubility of the citrate complex cannot account for the inhibition of citrate utilization by Mg<sup>++</sup>. The formation of soluble Mg<sup>++</sup> citrate complexes which cannot penetrate the cell membrane has been postulated (2).

Ochoa and Weisz-Tabori (3) showed that Mn<sup>++</sup> was necessary for the oxidative decarboxylation of oxalosuccinate. Kornberg *et al.* (4) demonstrated that Mg<sup>++</sup> could replace Mn<sup>++</sup> but only at higher concentrations; at concentrations of Mg<sup>++</sup> equal to the optimum for Mn<sup>++</sup>, the activity with Mg<sup>++</sup> was very low. Therefore, a competition between Mg<sup>++</sup> and Mn<sup>++</sup> for the enzyme system involved seemed plausible. The possibility that competitive inhibition was involved was further indicated by spectrophotometric studies showing that Mg<sup>++</sup> and Mn<sup>++</sup> both form keto acid complexes with the same relative affinity (4).

This study was undertaken in order to determine whether Mg<sup>++</sup> and other metallic ions could compete with Mn<sup>++</sup> for the enzyme systems which oxidize citrate. The results demonstrate this "competitive" effect when Mg<sup>++</sup> (or another metallic ion) is incubated with a dialyzed enzyme preparation a short while before the addition of Mn<sup>++</sup> and substrate.

<sup>1</sup> This work was supported by grants from the Nutrition Foundation, Inc., Smith, Kline and French, Inc., and the Office of Naval Research.

## METHODS

The enzyme system for the experiments was contained in a crude homogenate of rabbit kidney cortex. The cortex was removed immediately after the death of the rabbit, weighed and homogenized in isotonic NaCl-KCl saline at 0°C. in a Potter-Elvehjem ground glass homogenizer (5) for 3 min. The homogenate was then diluted with NaCl-KCl solution so that 1 ml. of the diluted homogenate contained 100 mg. wet weight of tissue.

The mitochondrial preparations were made essentially as described by Claude (6, 7), Hoogboom (8), and Schneider (9). Ten g. of the kidney cortex was homogenized as previously described. The homogenate was then centrifuged for 3 min. at  $1500 \times g$  to sediment the nuclei, whole cells, and debris from the mitochondria in the supernatant fluid. The sediment was washed with saline solution (2 parts 0.9% NaCl + 1 part 1.15% KCl) and again centrifuged for 3 min. at  $1500 \times g$ . The supernatant solutions were combined and centrifuged for 25 min. at  $2400 \times g$ , thus throwing down the mitochondria as a gray-white precipitate. These mitochondria were re-suspended in NaCl-KCl solution and centrifuged for 5 min. at  $6000 \times g$ . This wash was repeated 4 times, and the mitochondria were then dispersed in 20 ml. of NaCl-KCl solution making a suspension containing approximately 12 mg. dry weight/ml. This preparation was then dialyzed in a cellophane sack for 1 hr. against 20 l. of distilled water. The dialysis bag was tied over a slowly rotating bent glass rod in such a way that the dialysis bag turned end over end, thus agitating the contents of the bag as well as the dialysis water. All the above operations were carried out in a cold room at 0°C. Solutions of the metallic salts (reagent grade) were made with glass-redistilled water. Adenosine triphosphate was prepared according to the method described in *Biochemical Preparations* (10). Veronal buffer was prepared according to Michaelis (11). Sodium citrate (c. p.) was used as substrate. Citrate was determined according to the method of Saffran and Denstedt (12). Oxygen uptake was determined in the conventional Warburg apparatus at 30.1°C.

The mitochondrial preparations and reagents were measured into the Warburg vessels by means of calibrated micropipets, which are a modification of those used by Lindberg and Hummel (13).

## RESULTS

Homogenates of rabbit-heart-muscle oxidized citrate at approximately the same rate as did kidney cortex homogenates; liver homogenates were approximately one-half as active as heart muscle and kidney cortex, while homogenates of brain and skeletal muscle showed little or no activity under the same conditions. Since kidney cortex is more plentiful than heart muscle and is easier to work with, kidney cortex was employed in the following experiments.

The effects of varying concentrations of kidney cortex were examined. One hundred twenty-five mg. wet weight tissue per flask appeared to be the optimum concentration under the conditions of these experiments, and this amount of tissue was employed in the following experi-



ments. With increasing concentrations of citrate, up to 0.01 *M*, oxygen uptake increased. Higher concentrations had no further effect. Veronal at 0.01 *M* concentration was an effective buffer and was not toxic to the system.

After dialysis of the ground tissue preparation for 30 min., the necessity of  $Mn^{++}$  or  $Mg^{++}$  for citrate oxidation could be demonstrated in approximately the same optimum concentrations as originally reported by Adler *et al.* (14) (Table I). The optimum for  $Mn^{++}$  is approximately  $5 \times 10^{-5}$  *M* in this system, and that for  $Mg^{++}$ ,  $2.5 \times 10^{-3}$  *M*.  $Mg^{++}$  is completely inactive at a concentration of  $5 \times 10^{-5}$  *M*. Inorganic phosphate which is necessary for citrate oxidation to proceed is also removed on dialysis; activity can be restored on addition of varying concentrations of phosphate.

TABLE I

*Effect of Various Concentrations of  $Mn^{++}$  and  $Mg^{++}$  on the Oxidation of Citrate*

Tissue 125 mg wet weight per vessel, dialyzed 30 min., veronal buffer, pH 7.4, 0.01 *M*; phosphate, pH 7.4, 0.003 *M*; citrate 0.01 *M*;  $MnSO_4$  and  $MgSO_4$  in indicated concentrations, saline solution, total volume of 30 ml., plus KOH in alkali well, time, 1 hr.

$Mn^{++}$ concentration	Microliters $O_2$ uptake		$Mg^{++}$ concentration	Microliters $O$ uptake	
		Change due to $Mn^{++}$			Change due to $Mg^{++}$
<i>M</i>			<i>M</i>		
—	178	—	—	178	—
$5 \times 10^{-5}$	290	112	$1 \times 10^{-1}$	222	44
$2 \times 10^{-5}$	280	102	$2.5 \times 10^{-1}$	286	108
$5 \times 10^{-4}$	275	97	$1 \times 10^{-2}$	273	95
$2 \times 10^{-1}$	250	72			

Since  $Mg^{++}$  is active at higher concentrations but is inactive at lower concentrations (4),  $Mg^{++}$  could conceivably compete with  $Mn^{++}$  for the enzyme system involved. This competitive effect between  $Mg^{++}$  and  $Mn^{++}$  is demonstrated in Table II. The dialyzed tissue preparation had a very low endogenous oxygen uptake. On the addition of citrate, the oxygen uptake was increased and was further increased on the addition of  $Mn^{++}$  but not on the addition of  $Mg^{++}$ . When  $Mn^{++}$  ( $5 \times 10^{-5}$  *M*) was incubated with the dialyzed tissue for 15 min. and then  $Mg^{++}$  ( $5 \times 10^{-5}$  *M*) and finally citrate were added, the increased

oxygen uptake due to the presence of the metals was 101 and 109  $\mu$ l. in duplicate vessels (Table II).  $Mg^{++}$  without  $Mn^{++}$  had no effect. If  $Mn^{++}$  and  $Mg^{++}$  were added simultaneously to the dialyzed tissue preparation followed by the addition of citrate, there was no diminution in oxygen uptake. However, when  $Mg^{++}$  was incubated with the dialyzed tissue preparation for 15 min., and then  $Mn^{++}$  and finally citrate were added, the oxygen uptake due to the presence of the metals was inhibited 60–5% (Table II). Therefore,  $Mg^{++}$  at low concentrations cannot reactivate the dialyzed enzyme system but it also prevents  $Mn^{++}$  from reactivating it. It seems probable that  $Mg^{++}$  forms an inactive, stable complex with the dialyzed enzyme system, preventing the entrance of  $Mn^{++}$  into the system and thus inhibiting the activity of the enzyme. Since  $Mn^{++}$  is known to function in the decarboxylation of oxalosuccinic (3),  $\alpha$ -ketoglutaric (15), and oxalacetic acids (16), all of which are intermediates in the oxidation of citrate, this competitive effect can presumably take place at any one of these steps.

These competitive effects were obtained in every one of at least six experiments, with an occasional variation. In one or two experiments, the increased oxygen uptake on the addition of the metals was slightly less when both  $Mn^{++}$  and  $Mg^{++}$  were added simultaneously than when  $Mn^{++}$  was incubated with the tissue and  $Mg^{++}$  added afterward.

The possibility that  $Mg^{++}$  might exert its effect by precipitating inorganic phosphate as an insoluble magnesium phosphate was tested

TABLE II

*Competitive Effects of  $Mg^{++}$  and  $Mn^{++}$  in Citrate Oxidation*

Tissue (dialyzed 45 min.) 125 mg. wet weight per flask; veronal buffer, pH 7.4, 0.01 *M*; citrate 0.01 *M*; phosphate, pH 7.4, 0.003 *M*; plus saline solution to total volume of 3.0 ml.; KOH in alkali well.  $MnSO_4$  and  $MgSO_4$ ,  $5 \times 10^{-5}$  *M*, added as indicated. Time, 90 min.

Conditions	Microliters $O_2$ uptake	
Dialyzed tissue + additions; citrate in side arm		Change due to metal
No citrate	21	—
No metal	131	—
$Mn^{++}$ ; 15 min. later, $Mg^{++}$ added	232	101
$Mn^{++}$ ; 15 min. later, $Mg^{++}$ added	240	109
$Mg^{++}$	131	0
$Mn^{++}$ + $Mg^{++}$ added simultaneously	234	103
$Mg^{++}$ ; 15 min. later, $Mn^{++}$ added	168	37
$Mg^{++}$ ; 15 min. later, $Mn^{++}$ added	173	42

and eliminated. The standard texts on inorganic analysis lack adequate information on solubilities of magnesium phosphate. Therefore, a turbidimetric study was undertaken of the solubilities of Mn and Mg phosphates. The solutions duplicated those used in the previously described manometric experiments except that no tissue homogenate was present.  $Mg^{++}$  at concentrations up to  $0.01 M$ , in the presence of  $0.003 M$  phosphate, showed no turbidity and transmitted 100% of the light.  $Mn^{++}$  ( $5 \times 10^{-5} M$ ) plus phosphate ( $0.003 M$ ) also transmitted 100% of the light as did a solution containing  $Mn^{++}$  ( $5 \times 10^{-5} M$ ) plus  $Mg^{++}$  ( $5 \times 10^{-5} M$ ) plus phosphate ( $0.003 M$ ). Therefore, precipitation of phosphate could not be a factor in the competitive effects described. However, on increasing the concentration of  $Mn^{++}$  from  $5 \times 10^{-5} M$  to  $5 \times 10^{-3} M$ , in the presence of  $0.003 M$  phosphate, the per cent transmission decreased from 100% to 55%. The phosphates of Mg are therefore more soluble than those of Mn. In the same experiment, the addition of citrate to a tube containing  $5 \times 10^{-3} M$   $Mn^{++}$  plus  $0.003 M$  phosphate increased the transmission reading to 83%, compared to 55% without citrate, therefore demonstrating the binding of  $Mn^{++}$  by citrate. However, this binding effect cannot be a factor in these experiments, since the same competitive effects were obtained (after incubating  $Mg^{++}$  + dialyzed tissue) whether  $Mn^{++}$  addition was followed by citrate, or whether  $Mn^{++}$  and citrate were added simultaneously.

In an effort to purify the preparation and eliminate extraneous protein factors, the above mentioned mitochondria preparation was used in the following experiments. Dialysis of the washed mitochondria preparations was more effective in removing  $Mn^{++}$ , since a concentration of  $3 \times 10^{-3} M$   $Mn^{++}$  was needed for optimum oxygen uptake, with citrate as substrate (Table III). This is very similar to the optimum concentration of Mn ( $1.3 \times 10^{-3} M$ ) necessary for oxalosuccinate decarboxylation (3). Also, adenosine triphosphate was removed and had to be added back to a final concentration of  $1 \times 10^{-3} M$ . As with the dialyzed whole homogenate,  $Mg^{++}$  in a concentration of  $3 \times 10^{-5}$  or  $3 \times 10^{-4} M$  had no effect on oxygen uptake when  $Mn^{++}$  ( $3 \times 10^{-3} M$ ) was added first. However, when  $Mg^{++}$  was added to the mitochondria preparation before the addition of  $Mn^{++}$ , a definite inhibition of oxygen uptake and citrate utilization could be demonstrated every time (Table III). At least 50% inhibition of citrate utilization was recorded in each of four experiments.

The inhibition produced by adding  $Mg^{++}$  first, to the dialyzed

enzyme system, can be reversed by a sufficiently high concentration of  $Mn^{++}$ . Using the method of Lineweaver and Burk (17), the inhibition was shown to be competitive, within the concentrations used in all experiments in this report. Higher concentrations of  $Mn^{++}$  were markedly inhibitory (Fig. 1). This is not a classical situation where we

TABLE III

*Competitive Effects of  $Mn^{++}$  and  $Mg^{++}$  in Citrate Oxidation*

Each vessel contains 15 mg. dry weight of mitochondrial preparation;  $Mn^{++}$ ,  $3 \times 10^{-3} M$  unless otherwise indicated;  $Mg^{++}$ ,  $3 \times 10^{-3} M$  in all flasks except Nos. 8-9 ( $3 \times 10^{-1} M$ ); veronal buffer, pH 7.4, .01  $M$ ; phosphate, pH 7.4, .003  $M$ ; citrate .01  $M$ ; ATP .001  $M$ ; redistilled  $H_2O$  to total of 3 ml.; 10% KOH in alkali well;  $O_2$  uptake recorded for 60 min.

Flask number	Conditions Dialyzed tissue + additions; citrate in side arm	Microliters $O_2$ uptake		Inhibition	Micrograms citrate utilized		Inhibition
			Change due to metal			Change due to metal	
1	No Citrate	—	—	%	—	—	%
2	No metal	194	—	—	3880	—	—
3	$Mn^{++} 3 \times 10^{-3} M$	196	2	—	—	—	—
4	$Mn^{++} 3 \times 10^{-4} M$	318	124	—	—	—	—
5	$Mn^{++} 3 \times 10^{-3} M$	358	164	—	—	—	—
6	$Mn^{++}$ ; 15 min. later, $Mg^{++}$	358	164	—	4410	530	—
7	$Mg^{++}$ ; 15 min. later, $Mn^{++}$	257	63	61	3880	0	100
8	$Mn^{++}$ ; 15 min. later, $Mg^{++}$	354	160	—	4630	750	—
9	$Mg^{++}$ ; 15 min. later, $Mn^{++}$	274	80	50	4260	380	50
10	No metal	94	—	—	1630	—	—
11	$Mn^{++}$ ; 15 min. later, $Mg^{++}$	269	175	—	3380	1750	—
12	$Mg^{++}$ ; 15 min. later, $Mn^{++}$	129	35	80	2460	830	53
13	$Mn^{++}$ ; 15 min. later, $Mg^{++}$	231	127	—	3285	1655	—
14	$Mg^{++}$ ; 15 min. later, $Mn^{++}$	101	7	95	2385	755	55

are dealing with a substrate versus an inhibitor. In this case, we are dealing with a metallic cofactor which may be regenerated and which is toxic at higher concentrations. A more detailed mathematical representation of the system is being studied since the conventional form does not take these factors into account. Similar results, demonstrating a competitive inhibition, were obtained with  $Cu^{++}$  and  $Mn^{++}$ .

The competitive effects of  $Na^+$ ,  $Ca^{++}$ ,  $Zn^{++}$ ,  $Cu^{++}$  and  $Hg^{++}$  were also examined and found to produce greater than 40% inhibition of oxygen uptake (Table IV) when added to the Warburg vessel, 15 min.

before the necessary  $3 \times 10^{-3} M$   $Mn^{++}$ . When added alone, these concentrations of inhibitory metals had no effect on the  $O_2$  uptake, in relation to a blank with no metal (Table III and IV). The concentrations used were as follows:  $Hg^{++}$ ,  $3 \times 10^{-16} M$ ;  $Zn^{++}$ ,  $3 \times 10^{-6} M$ ;  $Ca^{++}$ ,  $3 \times 10^{-7} M$ ;  $Na^+$ ,  $3 \times 10^{-3} M$ ;  $Cu^{++}$ ,  $3 \times 10^{-8} M$ .

The inhibitory effects were not due to impurities in the redistilled water or to the addition of  $Mn^{++}$  after the enzyme had come into

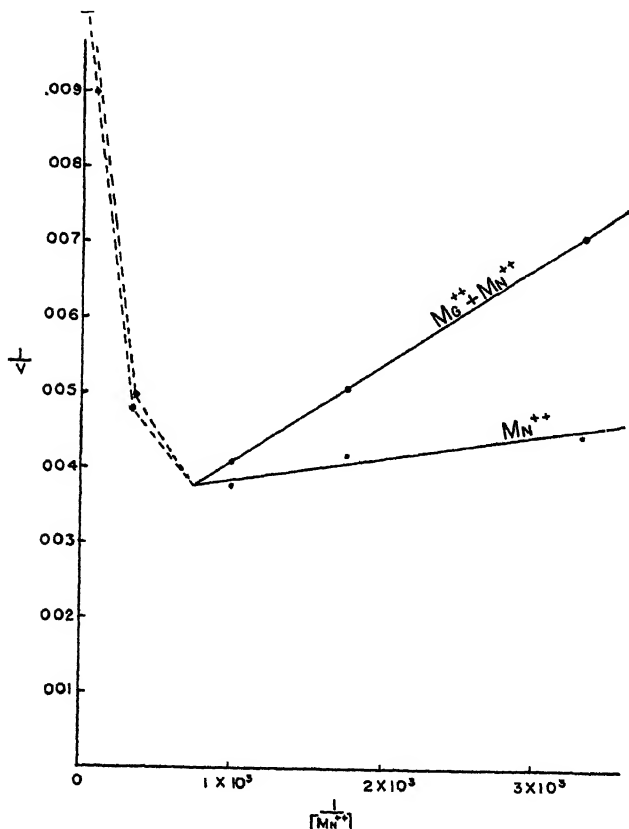


FIG. 1. Mitochondrial preparations, washed  $4 \times$  and dialyzed 60 min.; 0.01  $M$  citrate; 0.01  $M$  veronal buffer, pH 7.4; .003  $M$  phosphate; 0.001  $M$  ATP; made up to 3 ml. with redistilled water.  $\frac{1}{V}$  based on oxygen uptake for 1 hr.  $Mg^{++}$  concentration constant at  $3 \times 10^{-3} M$ ;  $Mn^{++}$  concentration varied as indicated. KOH in center well; temperature, 30°C.

TABLE IV

*Competitive Effects of Manganese and Various Metals in Citrate Oxidation*

Each vessel contains 15 mg. of mitochondrial preparation; veronal buffer, pH 7.4, .01 *M*; phosphate, pH 7.4, .003 *M*; citrate .01 *M*; ATP .001 *M*; redistilled water to volume of 3 ml.; 10% KOH in alkali well; O<sub>2</sub> uptake recorded for 60 min.

Conditions Dialyzed tissue + additions; citrate in side arm	O <sub>2</sub> uptake	Change due to metal	Inhibition %
No citrate	—	—	—
No metal	65	—	—
Mn <sup>++</sup> 3×10 <sup>-3</sup> ; 15 min. later, redist. H <sub>2</sub> O	166	101	—
Redist. H <sub>2</sub> O; 15 min. later, Mn <sup>++</sup> 3×10 <sup>-3</sup>	171	106	0
Mn <sup>++</sup> 3×10 <sup>-3</sup> ; 15 min. later, redist. H <sub>2</sub> O	200	135	—
Redist. H <sub>2</sub> O; 15 min. later, Mn <sup>++</sup> 3×10 <sup>-3</sup>	185	120	11
Hg <sup>++</sup> 3×10 <sup>-16</sup>	93	—	—
Mn <sup>++</sup> 3×10 <sup>-3</sup> ; 15 min. later, Hg <sup>++</sup> 3×10 <sup>-16</sup>	220	155	—
Hg <sup>++</sup> 3×10 <sup>-16</sup> ; 15 min. later, Mn <sup>++</sup> 3×10 <sup>-3</sup>	154	89	43
Mn <sup>++</sup> 3×10 <sup>-3</sup> ; 15 min. later, Hg <sup>++</sup> 3×10 <sup>-16</sup>	238	173	—
Hg <sup>++</sup> 3×10 <sup>-16</sup> ; 15 min. later, Mn <sup>++</sup> 3×10 <sup>-3</sup>	166	101	42
No metal	111	—	—
Mn <sup>++</sup> 3×10 <sup>-3</sup>	324	213	—
Zn <sup>++</sup> 3×10 <sup>-6</sup>	117	6	—
Mn <sup>++</sup> 3×10 <sup>-3</sup> ; 15 min. later, Zn <sup>++</sup> 3×10 <sup>-6</sup>	278	167	—
Zn <sup>++</sup> 3×10 <sup>-6</sup> ; 15 min. later, Mn <sup>++</sup> 3×10 <sup>-3</sup>	170	59	65
Ca <sup>++</sup> 3×10 <sup>-7</sup>	104	0	—
Mn <sup>++</sup> 3×10 <sup>-3</sup> ; 15 min. later, Ca <sup>++</sup> 3×10 <sup>-7</sup>	274	163	—
Ca <sup>++</sup> 3×10 <sup>-7</sup> ; 15 min. later, Mn <sup>++</sup> 3×10 <sup>-3</sup>	155	44	73
No metal	40	—	—
Na <sup>+</sup> 3×10 <sup>-3</sup>	41	1	—
Mn <sup>++</sup> 3×10 <sup>-3</sup> ; 15 min. later, Na <sup>+</sup> 3×10 <sup>-3</sup>	110	70	—
Na <sup>+</sup> 3×10 <sup>-3</sup> ; 15 min. later, Mn <sup>++</sup> 3×10 <sup>-3</sup>	26	-14	100
Cu <sup>++</sup> 3×10 <sup>-8</sup>	39	—	—
Mn <sup>++</sup> 3×10 <sup>-3</sup> ; 15 min. later, Cu <sup>++</sup> 3×10 <sup>-8</sup>	105	65	—
Cu <sup>++</sup> 3×10 <sup>-8</sup> ; 15 min. later, Mn <sup>++</sup> 3×10 <sup>-3</sup>	23	-17	100
No metal	90	—	—
Cu <sup>++</sup> 3×10 <sup>-7</sup>	95	5	—
Mn <sup>++</sup> 3×10 <sup>-3</sup> ; 15 min. later, Cu <sup>++</sup> 3×10 <sup>-7</sup>	200	110	—
Cu <sup>++</sup> 3×10 <sup>-7</sup> ; 15 min. later, Mn <sup>++</sup> 3×10 <sup>-3</sup>	144	54	50

contact with the buffer, phosphate, adenosine triphosphate, etc., since the addition of redistilled water (in place of inhibitory metallic ions) to the enzyme before or after the addition of Mn<sup>++</sup> had no effect (Table IV).

## SUMMARY

1.  $Mn^{++}$  and inorganic phosphate are necessary for the oxidation of citrate by a dialyzed rabbit kidney cortex homogenate.  $Mg^{++}$  may substitute for  $Mn^{++}$  if the concentration of  $Mg^{++}$  is increased to  $2.5 \times 10^{-3} M$ , 50 times the concentration of  $Mn^{++}$  ( $5 \times 10^{-5} M$ ).

2. If  $Mg^{++}$ , at a concentration of  $5 \times 10^{-5} M$ , is added to the dialyzed system prior to the addition of  $Mn^{++}$ , the  $Mg^{++}$  inhibits the oxidation of citrate. This inhibition by  $Mg^{++}$  is competitive and is not due to the precipitation of inorganic phosphate from the system nor to complex-formation of citrate with the metallic ions.

3. In experiments with washed and dialyzed mitochondria preparations obtained from rabbit kidney cortex, the optimum concentration of  $Mn^{++}$  for citrate oxidation was found to be  $3 \times 10^{-3} M$ . The following concentrations of metallic ions did not inhibit the oxidation of citrate when  $Mn^{++}$  ( $3 \times 10^{-3} M$ ) was added first to the tissue preparation:  $Mg^{++}$ ,  $10^{-5} M$ ;  $Hg^{++}$ ,  $3 \times 10^{-16} M$ ;  $Zn^{++}$ ,  $3 \times 10^{-6} M$ ;  $Ca^{++}$ ,  $3 \times 10^{-7} M$ ;  $Na^{+}$ ,  $3 \times 10^{-3} M$ ;  $Cu^{++}$ ,  $3 \times 10^{-6} M$ . However, when these metals in their indicated concentrations were added to the tissue preparation before the addition of  $Mn^{++}$ , inhibitions of the order of 40–100% of oxygen uptake and citrate utilization were obtained.

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# A Microbiological Study of *Cryptococcus Neoformans*<sup>1</sup>

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## INTRODUCTION

Cryptococcosis (torulosis) is a disease characterized by invasion of the body by a yeast-like organism known as *Cryptococcus neoformans*, *Torula histolytica*, or *Torula hominis* (1). This disease is uniformly fatal when the central nervous system is involved. The nutritional requirements of this organism were first determined in a chemically defined medium. Then the *in vitro* inhibitory action of certain compounds on this organism was studied in various media. Those compounds which exhibited therapeutic promise were then studied *in vivo* with infected mice.

## EXPERIMENTAL

### *Media, Cultures, Inoculum and Analytical Procedure*

A new procedure utilizing a chemically defined medium was developed for studying the nutritional requirements of this organism. Each liter of basal medium contained the following constituents (I): glucose, 20 g.;  $(\text{NH}_4)_2\text{SO}_4$ , 0.17 g.;  $(\text{NH}_4)_2\text{HPO}_4$ , 1 g.;  $\text{KH}_2\text{PO}_4$ , 1.5 g.;  $\text{NaCl}$ , 1 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3 g.;  $\text{CaCl}_2$ , 0.2 g.;  $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ , 1 mg.;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 1 mg.;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.2 mg.;  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ , 0.2 mg.;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.14 mg.;  $\text{KI}$ , 0.1 mg.; and molybdic acid powder (85%), 0.02 mg. (2,3).

Media containing the various growth factors, as indicated in I, were then prepared and adjusted to pH 6.7. Five-ml. portions were pipetted into  $18 \times 150$  mm. tubes, plugged with cotton, and autoclaved for 10 min. at 15 lb. pressure. Thirty-five cultures of *Cryptococcus neoformans* were available. Since qualitative studies of their cultural characteristics exhibited but minor variations,<sup>2</sup> 5 typical strains were

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<sup>2</sup> Observations of Drs. W. H. Mossberg, Jr., José A. Alvarez-De Choudens, and L. O. J. Manganiello, of the Department of Neurosurgery.



selected for further investigation. Ten ml. of beef broth solution were inoculated with a loopful of the pure culture taken from a well-grown Sabouraud's agar slant, incubated at 37°C. for 24 hr., and then at room temperature for 24 hr. The tube was then centrifuged, the fluid decanted and 10 ml. of sterile physiological NaCl solution added to the residue. The suspension was counted in a hemacytometer. The counts varied from 4500 to 5500 organisms/mm.<sup>3</sup> Each culture was then inoculated with a drop of this suspension and incubated in the dark at room temperature. Readings were made at convenient intervals over a 4-week period.

Growth response was measured by acid production and by turbidity. To 5 ml. of the basal medium was added a trace of dry starch which produced a turbidity resembling that in the culture tube. Five to 7 drops of bromothymol blue indicator was added and the pH adjusted to 6.9. This tube was used as a standard for comparison. After addition of the indicator (4), each assay tube was then titrated to pH 6.9 with 0.02 *N* sodium hydroxide. Care was taken to prevent self-infection.

The titrated, cotton-plugged tubes were then sterilized at 18 lb. steam pressure for 15 min. The heavy, white, adherent cell growth was thereby reduced to a fine suspension. Two drops of 1 *N* sulfuric acid were added to each tube and the volumes adjusted to 15 ml. with water. A tube of basal medium treated as above (except for the omission of starch) was set at 0 in a Klett-Summerson photoelectric colorimeter which contained the No. 66 red-light filter (640–700  $m\mu$ ). The contents of each tube were mixed by inversion and read immediately. Serial dilutions of suspensions gave straight-line curves.

## RESULTS AND DISCUSSION

Since only minor differences among the activities of the 5 strains studied were observed, averaged acidity and turbidity readings for the 5 strains are reported. The data in Table I indicate clearly that the organism grows vigorously in a solution containing glucose, inorganic salts, ammonium salts, and vitamins. Purines and pyrimidines are not essential and amino acids exert merely a slight stimulatory effect. A few tubes were incubated at 37°C. Although initial growth was somewhat faster, final acid production and turbidity were lower than the tubes incubated at room temperature.

The data in Table I also show that the addition of thiamine alone to the basal medium resulted in an increase in acidity and turbidity equal to that given by a mixture of all vitamins. Addition of each of the other vitamins singly to the basal medium did not stimulate growth; also, omission of vitamins singly, other than thiamine, from the vitamin composite did not decrease production of acid or growth (data omitted). Burkholder and Moyer (5) studied the vitamin requirements of 33 yeasts and 17 molds, including certain pathogens, in chemically defined media. Biotin and thiamine deficiencies occurred most commonly, although deficiencies for pyridoxine, pantothenic acid,

TABLE I  
Nutritional Requirements of *Cryptococcus neoformans*

Days after inoculation	Acid production as ml. of 0.02 N NaOH/5 ml. of medium								Growth rate as Klett-Summerson turbidimetric readings							
	Basal medium	Basal + amino acids <sup>a</sup>	Basal + all vitamins <sup>b</sup>	Basal + thiamine chloride <sup>c</sup>	Basal + vitamins + purines + pyrimidines <sup>d</sup>	Basal + vitamins + amino acids	Basal + vitamins + purines + pyrimidines	Basal + vitamins + purines + amino acids + pyrimidines	Basal medium	Basal + amino acids <sup>a</sup>	Basal + all vitamins <sup>b</sup>	Basal + thiamine chloride <sup>c</sup>	Basal + vitamins + purines + pyrimidines <sup>d</sup>	Basal + vitamins + amino acids	Basal + vitamins + purines + amino acids + pyrimidines	Basal + vitamins + purines + amino acids + pyrimidines
0	0.7	0.9	0.9	0.7	0.9	1.0	0.9	2	2	2	2	2	2	2	2	2
3	0.9	1.1	1.3	1.2	1.3	1.4	1.3	9	19	23	18	23	29	37	37	37
7	0.9	1.3	2.4	2.1	3.0	3.2	2.9	5	13	35	33	39	40	48	48	48
10	1.0	1.5	3.3	3.1	3.9	4.6	4.3	9	16	45	44	51	70	66	66	66
14	1.2	1.5	4.6	4.0	4.5	5.4	4.7	6	16	64	62	71	70	61	61	61
17	1.2	1.7	4.8	4.7	5.0	5.2	5.2	11	16	74	100	104	97	125	125	125
21	1.3	1.5	5.0	4.8	4.9	5.6	5.5	10	26	103	100	112	149	171	171	171
24	1.2	1.9	5.3	5.1	5.4	6.2	5.3	6	24	121	114	133	176	165	165	165
28	1.2	2.1	5.8	5.3	5.6	6.2	5.6	10	23	128	125	154	185	177	177	177

<sup>a</sup> 200 ml. of basal medium contained 1.2 ml. of 10% casein hydrolyzate and 20 mg. of L-tryptophan.

<sup>b</sup> 200 ml. of basal medium contained inositol, 800, choline chloride, 200, p-amino benzoic acid, 120, pyridoxine, 120, pyridoxamine, 50, pyridoxal, 50, thiamine chloride, 80, niacin, 40, calcium pantothenate, 40, riboflavin, 20, biotin, 0.6, and synthetic folic acid, 0.4 µg., respectively.

<sup>c</sup> 200 ml. of basal medium contained 2 mg. of adenine, guanine, uracil, thymine, and xanthine, respectively.

<sup>d</sup> 200 ml. of basal medium contained 80 µg. of thiamine chloride.

inositol, and nicotinic acid were found in several yeasts. While the present work was in progress, Hehre, Carlson, and Hamilton (6) isolated a crystalline amylose produced by *Torula histolytica* on a synthetic medium containing only thiamine as a source of vitamins.

Increasing increments of thiamine chloride added to 5-ml. portions of basal media resulted in a progressive increase in acidity and turbidity. Thus, after 23 days of incubation, the tubes containing 0, 0.002, 0.01, 0.05, and 0.15  $\mu\text{g.}$  of thiamine chloride required the following volumes for titration to pH 6.9: 1.3, 1.8, 2.3, 4.1, and 5.9 ml. of 0.02 *N* sodium hydroxide, respectively; the corresponding Klett-Summerson turbidity readings were 15, 39, 59, 85, and 113, respectively.

The organism readily combines the pyrimidine (2-methyl-5-ethoxymethyl-6-amino-pyrimidine) and thiazole (4-methyl-5-thiazoleethanol) moieties to form thiamine. Thus the addition of 0.5 and 0.05  $\mu\text{g.}$  of each moiety to 5-ml. quantities of basal media gave titration and turbidity values, after incubation for 23 days, of 6.0 and 6.1, and 109 and 110, respectively—values equivalent to those obtained with thiamine. The organism grew poorly when only the pyrimidine portion of thiamine was added to the medium. However the addition of 1 and 0.1  $\mu\text{g.}$  of the thiazole moiety to 5 ml. portions of basal media gave readings of 3.8 and 3.0, and 85 and 77, respectively, after 23 days of incubation at room temperature. Hence the organism can synthesize the pyrimidine but not the thiazole part of the thiamine molecule.

Oxythiamine has been reported to be an active thiamine inhibitor (7,8). An oxythiamine solution was sterilized by passage through a Morton bacterial filter and 0.2, 2.0, and 20  $\mu\text{g.}$  of oxythiamine was added to 5-ml. portions of sterile basal media. After inoculation and incubation for 24 days, the acidity and turbidity readings were 2.4, 3.7, and 5.7, and 65, 95, and 100, respectively. Thus, greater amounts of oxythiamine are required to produce growth equal to that obtained with thiamine. However, our data yield no information as to the mode of oxythiamine utilization by the organism.

The addition of 4, 20, 100, 500, 2000, and 4000  $\mu\text{g.}$  of oxythiamine (sterilized by filtration) to tubes each containing 5 ml. of sterile basal media and 1  $\mu\text{g.}$  of thiamine chloride did not produce growth inhibition. The titration values obtained were 6.8, 6.7, 6.4, 6.8, 6.3, and 6.2. The corresponding turbidity readings were 110, 100, 126, 99, 110, and 110, respectively.

The thiamine inhibitor pyrithiamine (9,10,11), as prepared by the method of Tracy and Elderfield (12), has been shown by Wilson and Harris (13) to be a mixture of pyridine and pyrimidine moieties. These workers have apparently obtained the correct analog which they have named neopyrithiamine. A solution of this compound (Merck and Company), which was sterilized by filtration and added to 5-ml. portions of sterile basal media in amounts ranging from 1-1000  $\mu\text{g.}/\text{ml.}$  of medium, did not support growth. We also found that neopyrithiamine did not effectively inhibit the utilization of added thiamine by the organism in the liquid medium. Thus, when 0.1, 1, 10, 160, 800, and 4000  $\mu\text{g.}$  were added to tubes each containing 5 ml. of sterile basal medium and 1  $\mu\text{g.}$  of thiamine chloride, the acidity and turbidity readings were 6.5, 6.2, 5.7, 3.1, 3.0, and 3.5, and 121, 90, 98, 77, 80, and 73, respectively, after the usual period of incubation. Wyss (14) concluded that pyrithiamine shows little promise as a systemic antibacterial agent.

#### *In Vitro Inhibition of Cryptococcus neoformans in Liquid Medium*

Accurately weighed quantities of various compounds were dissolved in water or alcohol. Appropriate dilutions were then prepared and 5-ml. quantities added to 50-ml. portions of basal medium which also contained the mixed vitamins (see Table I b). Five-ml. portions were tubed, sterilized, inoculated, and incubated as usual. While readings were made at 5-day intervals, only those made after 24 days of incubation are reported in Table II.

The most effective inhibitors by this procedure were the vitamin K derivatives synkamin (4-amino-2-methyl-1-naphthol hydrochloride), hykinone (menadione sodium bisulfite), synkayvite (2-methyl-1,4-naphthalenediol diphosphate tetra-sodium salt) and the antibiotics actidione and tomatin. These compounds are known to possess anti-microbial activity (15-20). Biacetyl, chlorothymol, dithiobiuret, and the quaternary ammonium compounds were also effective inhibitors. The remaining compounds examined were either inactive or but slightly fungistatic. Due to technical difficulties, this procedure was abandoned in favor of the agar diffusion procedure.

#### *In Vitro Inhibition of Cryptococcus neoformans in Agar Medium*

Sabouraud's agar (6.5 g./100 ml. of water) was dissolved in hot water and 20-ml. portions transferred to test tubes and sterilized for 15 min. at 15 lb. pressure. The various compounds were dissolved in water, alcohol, propylene glycol, or mixtures of

TABLE II  
*Inhibition of Cryptococcus neoformans in Liquid Medium*

Drug studied	Concentration of drug per ml. of medium									
	2 mg./ml.		0.2 mg./ml.		0.04 mg./ml.		0.02 mg./ml.		0.004 mg./ml.	
	a	b	a	b	a	b	a	b	a	b
Menadione sodium bisulfite	0.8	9	0.7	6	0.7	6	0.6	6	4.7	107
4-Amino-2-methyl-1-naphthol-HCl	0.5	4	0.8	8	0.5	4	1.3	30	4.4	83
1,4-Naphthalenediol-2-methyl diphosphate tetra-sodium salt	0.8	4	1.8	9			1.5	8	4.5	83
Actidione	1.5	8	1.7	10	1.7	20	4.5	112	5.1	130
Tomatin	1.1	8	1.7	10	1.0	8	5.0	110	6.0	105
Dimethyl benzyl higher alkyl ammonium chloride		13		12		10		18		90
Cetyl trimethyl ammonium bromide		7		16		11		3.7	33	
Chlorothymol	1.6	6	0.7	7	0.8	15	2.0	25	5.0	90
Thymol	1.5	5	2.0	19	4.1	62	4.1	66		
Carvacrol	1.7	4	2.5	25	4.7	60				
Eugenol	1.8	6	2.0	20	5.0	80	5.0	55		
Dithiobiuret	1.0	5	1.0	5	2.0	30	5.2	82		
Ethylene thiourea	2.1	4	3.1	104	3.8	95				
Ethylthiourea	3.0	50	5.0	111	5.0	111				
Canavanine sulfate	5.8	140	5.9	135						
Pyridine-3-sulfonic acid	6.0	145	5.7	120						
$\beta$ -Alanine	5.0	111	5.3	120						
Uninoculated control	1.0	4	0.9	2	1.2	5				
Inoculated control	5.8	130	6.8	125	6.1	130	5.8	135	6.0	125

<sup>a</sup> ml. 0.02 *N* sodium hydroxide.

<sup>b</sup> Klett-Summerson turbidimetric readings.

the same. Appropriate dilutions were then made, preferably with water, although the other solvents do not influence growth of the organism. These solutions were not sterilized. Then 0.5 ml. of each drug solution was mixed with 20-ml. portions of hot agar and poured into sterile Petri dishes. The solutions were of such concentration that each plate contained 2.5, 0.25, 0.025, 0.005, or 0.001 mg. of drug, respectively, per milliliter of agar medium. The cooled plates were inoculated with 4 radial streaks by means of a sterile loop and a saline suspension of active organisms. Plates, including controls, were prepared in duplicate and inspected visually for growth 5 and 10 days after inoculation. Growth was luxuriant and appeared glistening, mucoid, and slightly tan in color. Adequate concentration of effective drugs produced complete inhibition of growth. Occasional contamination caused no difficulties in the reading

of the plates. The data have been arranged according to the concentration of drug per milliliter of agar medium which produced complete inhibition of growth for 10 days.

*0.005 mg. of drug/ml. of agar medium.* Synkamin, dithiocyanoacetanilide, hexachlorophene, methyl-5-nitro-2-furoate, 8-hydroxyquinoline, dimethyl dichlorosuccinate, cetyl dimethyl ethyl ammonium bromide, and cetyl trimethyl ammonium bromide.

*0.025 mg. of drug/ml. of agar medium.* Biacetyl, pseudomethyl acetylacrylate, eschridine [4-(4'-ethylcyclohexylmethyl) pyridine], germitol (dimethyl benzyl higher alkyl ammonium chloride), dimite [di(*p*-chlorophenyl)methyl carbinol], 1-lauryl-3-methyl and 1-lauryl-4-methyl pyridinium chloride, sulfapyridine, dithiocyananiline, 4-thiocyano-2-nitroaniline, methyl ester of thiocyananthranilic acid, dinitrophenol, butadiene dithiocyanate, chlorothymol, bis(5-chloro-2-hydroxyphenyl) methane, benzyl *p*-hydroxybenzoate, undecylenic acid, phenosafranin, methyl violet, brilliant violet, and sodium azide.

*0.25 mg. of drug/ml. of agar medium.* Acridine, thymol, carvacrol, octyl resorcinol, *p*-chlorobenzoic acid, thiocyanacetanilide, hexamethylenetetramine thiocyanate, thiocyananiline, dithiobiuret,  $\alpha$ -naphthylamine, methacrylic acid, acetophenone-oxime, isonitrosopropiophenone, anesthesin, 3-myristoyl aminopropyl dimethylbenzyl ammonium chloride, zinc undecylenate, 2,6-dibromoquinonechloroimide, bromothymol blue, 5-nitro-2-furaldehyde diacetate, and methyl-5-nitro-2-furfuryl ether.

*2.5 mg. of drug/ml. of agar medium.* Furfural, hydrofuramide, acetophenone, ketophenyl ethylamine·HCl, isophorone, *p*-hydroxybenzaldehyde, *p*-cresol, thiocresol, vanillin, propionic acid, coumarin, epichlorohydrin, acetanilide, aniline, butyn sulfate, pyridine-3-sulfonic acid, indole-3-carboxylic acid, *n*-allylthiourea, thiourea, butyl thiourea, aminophyllin, 5-nitro-2-furfuraldehyde semicarbazone, diaminoacridine sulfate, neutral red, methylene blue, thioglycolic acid, *N*-2-chlorophenylphthalamic acid, *N*-1-naphthalamic acid, 2,4-dichlorophenoxy acetic acid, dicoco dimethyl ammonium chloride, dihydrogenated tallow dimethyl ammonium chloride, 7-hydroxy-4-methyl coumarin, *p*-[bis-(carboxy-methylmercapto)arsino]benzene sulfonamide, and *p*-[bis-(carboxy-methylmercapto)arsino]benzamide.

*Inactive.* Bacitracin, aureomycin, chloromycetin, subtilin, *d*-usnic acid, *p*-aminobenzoic acid, sodium sulfathiazole, sulfamerazine, sulfapyrazine, sulfamethazine, sodium sulfadiazine, sulfamylon, 3,4-dimethyl-5-sulfanilamidoisoxazole, darvisul [*N*-(2-thiazolyl)-phenol sulfonamide], 2-sulfanilamidoquinoxaline, *p*-aminosalicylic acid, *dl*- $\alpha$ -tocopherol acetate, menadione sodium bisulfite, 1,4-naphthalenediol-2-methyl diphosphate tetra-sodium salt, A-methopterin, hematoporphyrin·HCl, sulfanilic acid, sodium taurocholate, furacin, 1-diethylcarbaryl-4-methylpiperazine dihydrogen citrate, gallocyanine,  $\alpha$ -tetradecylbutyrolactone- $\alpha$ -carboxylic acid, diacetone alcohol, acetonyl acetone, propylene glycol, phloroglucinol, pentanediol, mesityl oxide, ditertiary butyl peroxide, phenurone, acetophenanthalide, methyl acrylate, sodium-1,2-naphthoquinone-4-sulfonate, dimethylglyoxime, *d*-camphoric acid, glutathione, alizarine red, methyl red, methyl orange, pyridine, 2-amino pyridine, 2-amino-3-methyl pyridine,  $\alpha$ -picoline, diacetyl-*p*-aminophenol, 4-pyridylpyridinium dichloride, *p*-hydroxyphenyl arsonic acid, 6,9-dichloro-2-methoxyacridine, maleic hydrazide, ethylenediamine tetra-acetic acid, 4-carbamidophenyl

di-(carboxy-methyl-thio)arsenite, morphyline thiocyanate, *m*-thiocyanobenzoic acid, potassium thiocyanate, 6-carboxy-3-pyridinesulfonic acid, and 5-nitro-2-furfuraldehyde-2-(2-hydroxyethyl semicarbazone).

The vitamin K derivative synkamin was especially effective in inhibiting growth of the organism in both liquid and agar media. This inhibitory activity could be completely reversed by the addition of cysteine or thioglycolic acid but not by methionine or succinic acid. Although hykinone and synkayvite were potent inhibitors in the liquid medium, both were entirely inert in the agar medium. Schwartzman (15) found that synkayvite possessed no antibacterial activity under his experimental conditions and that the potency of hykinone was distinctly lower than that of synkamin. Biacetyl and the quaternary ammonium compounds were also found to be effective inhibitors. Among the various nitrofurane derivatives which were examined, including furacin (21), only methyl-5-nitro-2-furoate was actively fungistatic. Likewise the dithiocyanates, especially dithiocyanacetanilide, were found to be more fungistatic than the monothiocyanates. Pseudomethyl acetylacrylate, hexachlorophene, 8-hydroxyquinoline, dimethyldichlorosuccinate, and sodium azide also prevented growth of the organism. While the higher alkylpyridinium compounds were inhibitory, small-molecule pyridine derivatives such as 2-aminopyridine, etc., were inert. Antibiotics such as aureomycin, chloromycetin, bacitracin, etc., were also inert, whereas actidione and tomatin (20) were active inhibitors of this organism. Sulfonamides were found to be noneffective, with the exception of sulfapyridine which is known to be without value in human torulosis. Organic arsenicals were also relatively inactive. Certain analogs of amino acids and essential growth factors including indole-3-carboxylic acid,  $\beta$ -alanine, A-methopterin (4-amino- $N^{10}$ -methyl pteroylglutamic acid) (22), and pyridine-3-sulfonic acid were also found to be relatively noninhibitory. The tuberculostatic compounds *p*-aminosalicylic acid and *d*-usnic acid (23) were also useless. Whereas phenosafranin, methyl violet, and brilliant violet exhibited moderate fungistatic activity, other dyes were found to be inert. Neopyrithiamine partially inhibited growth of the organism in the agar medium.

### *Pharmacotherapeutics*

The course of human torulosis, except for occasional temporary remissions, is one of steady progression and all attempts to combat the disease have proven unsuccessful (1,21,24,25,26). Failure is thought

to be due, mainly, to the difficulty of drug penetration into the central nervous system and through the thick polysaccharide capsule which surrounds the organism.

Certain compounds which were potent inhibitors *in vitro* appeared worthy of *in vivo* studies. The LD 50 of each of these compounds was determined according to the method of Trevan by intraperitoneal injection into mice. Fifty animals were used for each test. Compounds insoluble in normal saline were dissolved in propylene glycol or warm olive oil. The LD 50 for each of the following compounds was found to be as follows: biacetyl, 1320; methyl-5-nitro-2-furoate, 220; eschridine, 65; pseudomethyl acetylacrylate, 44; dithiocyanoacetanilide, 40; and hexachlorophene, 18 mg./kg. body weight, respectively. The values for synkamin and mapharsen were obtained from the literature.

Anesthetized mice were infected by intracerebral injection<sup>2</sup> of a saline suspension of the organisms. The therapeutic dose employed was based upon the LD 50 of each compound. Approximately one-third of the LD 50 per mouse was considered to be the maximum dose and was given daily by intraperitoneal injection. Doses of decreasing concentration were given to 3 other groups of infected mice. Each group contained 10 mice. Control experiments were also carried out.

After about 5 days, all infected mice, including the treated groups, became obviously ill and, at the tenth day, their skulls showed a swelling which later became spindle-shaped. This deformity is a definite indication of torulosis in mice.<sup>2</sup> All infected mice died within 4 weeks irrespective of the type or amount of drug injected, and post-mortem examination revealed infection localized in the central nervous system. A pathological examination of the animals showed that the organisms did not spread into other organs, due to rapid fatal meningo-encephalitis. Uninfected mice receiving the various drugs remained alive and well. There was no apparent evidence that the symptoms or progress of the disease was influenced by any of the drugs.

#### ACKNOWLEDGMENTS

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#### SUMMARY

*Cryptococcus neoformans* was grown successfully in a liquid medium containing glucose, ammonium and inorganic salts, and thiamine. The



inhibitory action of a large series of compounds was studied *in vitro* in liquid and in agar media. The injection of certain growth inhibitors exhibiting therapeutic promise failed to influence the course of experimental torulosis induced in mice.

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# The Stimulation of the Non-Enzymatic Decarboxylation of Oxalacetic Acid by Amino Acids <sup>1</sup>

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## INTRODUCTION

Although there has been considerable interest in the role of inorganic cations in the decarboxylation of oxalacetic acid (OAA) (1,2,3), there has been little attention paid to the possible role of the naturally occurring amines. It is well known that aniline exerts a marked stimulation on the spontaneous decarboxylation of OAA, but there are only traces of analogous aromatic amines in the tissues. In tissue experiments on the relation of glycine to the utilization of OAA by liver slices, it was noted that OAA disappeared more than twice as rapidly in the presence of glycine than in the control (4). In order to isolate this phenomenon the role of glycine in the spontaneous decarboxylation of OAA was studied. It was found that glycine, at a concentration of 0.05 *M*, stimulated the spontaneous decarboxylation to about five times the control rate. The investigation was extended to other amino acids and naturally occurring amines.

## EXPERIMENTAL

The conditions of the experiments were essentially those of Speck (2), with the exception that many of the reactions were carried out at 37°C. The final acetate buffer concentration was 0.05 *M*, pH 5.0. An air atmosphere was used throughout all of the experiments. About 20  $\mu$ moles of OAA was added to the side arm of each Warburg flask as a freshly prepared solution, which was tested for OAA content in a control flask containing nickel sulfate (5) or aniline acetate in a final

<sup>1</sup> This study was made in part under contract with the Office of Naval Research, and under a grant from the International Minerals and Chemical Corporation.

concentration of .01 *M*. The solution of reagent grade amino acid or inorganic salt to be tested, adjusted to pH 5.0 (glass electrode), was placed in the main compartment of the flask with the acetate buffer, and the reaction started by tipping, after a 10 min. equilibration period. Readings were taken at 10-min. intervals for the slower reactions, and at shorter intervals for the more rapid reactions. First-order reaction rates were calculated using the amount of OAA found in the control flask as the amount present in all of the flasks at zero time.

## RESULTS

It was found that all of the amino acids tested caused some increase in the non-enzymatic decarboxylation of OAA, and that they varied considerably in their effectiveness (Table I). The most active of the

TABLE I

*Catalytic Effect of Some Amines on the Decarboxylation of OAA*

Final concentration of added amine, 0.05 *M*; acetate buffer, pH 5.0, 0.05 *M*; temperature 37°C., except as noted. First-order rate constants<sup>a</sup> expressed as min.<sup>-1</sup>.

	<i>K</i>		<i>K</i>
Blank	.010	L(+) Glutamic acid	.026
Glycine	.049	Glutamine	.037
Acetylglucine	.018	Acetylglutamic acid	.013
DL-Alanine	.018	DL-Phenylalanine	.033
β-Alanine	.024	L(-) Tryptophan	.030
DL-Valine	.024	L(+) Histidine HCl (0.025 <i>M</i> )	.125
DL-Norvaline	.020	L(-) Proline	.015
L(-) Leucine	.025	L(-) Hydroxyproline	.020
DL-Norleucine	.026	Adenine sulfate	.023
DL-Isoleucine	.020	Guanidine carbonate	.012
DL-Serine	.031	Creatine	.009
DL-Threonine	.032	Creatinine	.010
DL-Ornithine HCl	.076	Urea	.012
L(+) Lysine HCl	.050	Ammonium chloride	.014
L(+) Arginine HCl	.053	Acetamide	.009
DL-Aspartic acid	.020	Choline chloride	.009
Asparagine	.040	<i>p</i> -Aminobenzoic acid	.182
Blank 30°C.	.006	<i>p</i> -Aminobenzene sulfonamide	.077
Aniline 30°C.	.341		

$$^a K = \frac{2.3}{t_2 - t_1} \log \frac{\text{OAA}_1}{\text{OAA}_2}$$

amino acids tested was L(+) histidine, which stimulated the decarboxylation approximately twenty-four fold at 30°, at .05 *M*. A similar stimulation at 37° could be determined by extrapolation (Fig. 1). The kinetics of all of the amines tested conformed to a first-order reaction with respect to OAA under the conditions of these experiments.

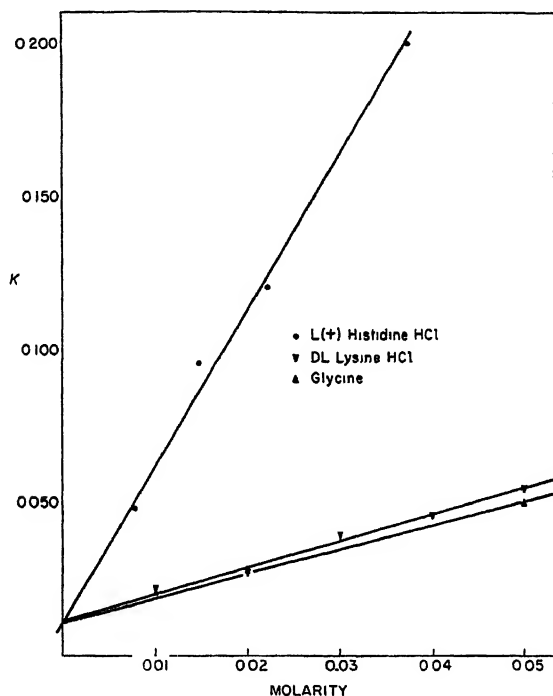


FIG. 1. Relation between concentration and catalytic effect of three amino acids. *K* represents a first-order reaction-rate constant, min.<sup>-1</sup>. The temperature was 37°C. For other conditions, see text.

The possibility that the effects noted might be caused by traces of metals contaminating the amino acids was tested in the following manner. Three of the most active amino acids: lysine, glycine, and histidine, were recrystallized twice from water and alcohol, and they were tested for activity after each recrystallization. The differences between the recrystallized and starting materials were within the limits of the experimental error for glycine and histidine. The constant

for lysine diminished by 15% on the first recrystallization, but there was no further diminution on subsequent recrystallization. As a further check on the possibility of metal contamination, various amino acids were tested in the presence of several multivalent cations which have been reported (1,2) as highly active in catalyzing the non-enzymatic and enzymatic decarboxylation of OAA. Only in the case of glycine and manganese was the effect of the metal ion and amino acid the sum of the catalytic effect of both. The addition of histidine inhibits the catalytic action of  $\text{Ni}^{++}$  and  $\text{Al}^{++}$  (Table II). Since mercury precipitation is the method by which L(+) histidine is prepared, it would be the most likely metallic contaminant. As can be seen from Table II, all the cations tested, including mercury, inhibit the effect of histidine. These results show that if the catalytic activity of the various amino acids were due to metallic contaminants they would have to comprise considerably more than the 0.1-.2% ash content of these amino acids.

In view of the reports (3,4) that ionic strength affects the enzymatic decarboxylation of OAA, the question of whether the hydrochloride moiety of the amino acid hydrochlorides used had any influence in the results reported was considered. Upon neutralization, this hydrochloride radical would yield additional ionic strength equal to the amino acid concentration. This was tested by adding increasing concentrations of sodium chloride to OAA with and without .05 *M* glycine. In the range tested, final concentrations of sodium chloride .05-.25 *M*, there was no appreciable effect on either the spontaneous decarboxylation of OAA, or on the glycine-catalyzed reaction.

It is to be noted from Table I that the primary amines are the most active, and that when the amino group is part of a resonating structure, as in guanidine, it loses its activity. Furthermore, the activity of the amino acid is related to the polarity of the beta substituent. Amidation of the dicarboxylic amino acids increases their activity, and acetylation of the amino group diminishes the activity. Although the relative activity of the amino acids in the decarboxylation of OAA is not exactly the same as the sequence observed by Langenbeck (6) in the catalytic effect of the amino acids on the decarboxylation of phenylglyoxylic acid, the same general rule seems to apply, namely, the catalytic activity of a substance in a homologous series increases with the number of polar groups and complexity of the structure. The only exception to this rule is glycine.

The spontaneous decarboxylation of the OAA we prepared according to the method of Krampitz and Werkman (7) had a constant of .010 min.<sup>-1</sup> at 37°, and .006 min.<sup>-1</sup> at 30°C., which is the value obtained by Vennesland *et al.* (5) (Table I).

The effect of three of the most active amino acids: glycine, L(+) lysine monohydrochloride, and L(+) histidine monohydrochloride, was directly proportional to the concentration in the range 0.01-.05 *M* (Fig. 1).

TABLE II

*Combined Effect of Multivalent Cations and Amino Acids on Non-Enzymatic Decarboxylation of OAA*

Column *K* represents the first-order rate constant for the cation alone, and the next two columns represent the constants for mixtures of cation and 0.05 *M* glycine and L(+) histidine monohydrochloride, respectively. The experiments were conducted at 30°C., the other conditions the same as for Table I. At this temperature the constant for 0.05 *M* glycine is 0.029, and for 0.05 *M* histidine 0.144. All constants are expressed in min.<sup>-1</sup>. The salts used were sulfates of Al<sup>+++</sup>, Ni<sup>++</sup>, and Mn<sup>++</sup>, FeCl<sub>3</sub>, and Hg(NO<sub>3</sub>)<sub>2</sub>.

Cation	<i>K</i>	Glycine	Histidine
Fe <sup>+++</sup> 0.10 <i>M</i>	.051 <sup>a</sup>	.062 <sup>a</sup>	.078 <sup>a</sup>
Al <sup>+++</sup> 0.001 <i>M</i>	.191	.194	.122
Ni <sup>++</sup> 0.001 <i>M</i>	.095	.101	.113
Hg <sup>++</sup> 0.01 <i>M</i>	.006	.014	.091 <sup>a</sup>
Mn <sup>++</sup> 0.01 <i>M</i>	.076	.102	

<sup>a</sup> These constants were not linear, indicating that the reactions were not first order (2). They represent the average constants over the range of OAA concentration from 90% to 10% of initial value.

Of the three aromatic amines tested: aniline, *p*-amino benzoic acid, and *p*-amino benzene sulfonamide, aniline is by far the most active catalytically (Table I). In comparing the relative activity of these compounds it was found that concentrations of citrate in the range used in the present manometric aniline method for the estimation of OAA (8) are markedly inhibitory of both aromatic and aliphatic amines. The effect is marked at concentrations of citrate as low as 0.01 *M*, at which concentration the catalytic effect of histidine is 85% of its activity in acetate buffer 0.05 *M*. This inhibitory effect can also be demonstrated with aniline. The inhibitory effect of low pH (1) was ruled out in these experiments, for they were all conducted at pH 5.0.

In view of the report of Shishova (9) that carnosine stimulates the decarboxylation of OAA by frog muscle extracts, we prepared carnosine, m. p. 259–61° (uncorrected) with decomposition, from veal according to the method of Dietrich (10). At .05 *M*, carnosine exerted very little stimulatory effect on the spontaneous decarboxylation (Table I).

### DISCUSSION

The catalytic effect of amines on the decarboxylation of keto acids has been studied extensively. Although the substrates have been mainly synthetic, such as camphoglyoxylic (11), bromocamphocarboxylic (12), and camphocarboxylic (13) acids, some studies have been done demonstrating this catalytic effect on acetoacetic (14,15), acetonedicarboxylic (16), and oxalacetic (17) acids. Pollak (15), after demonstrating that serum and organ extracts stimulated the decarboxylation of acetoacetic acid, showed the catalytic effect of hydrolyzed serum and peptones, and also of several amino acids. Mayer (18) observed the same effect with precipitated yeast protein. Langenbeck's extensive studies on carboxylase models (6) included some of the amino acids, but those experiments were done on synthetic substrates. Breusch (19), in investigating the nature of a heat-stable, water-soluble extract of various tissues which he found to stimulate the decarboxylation of OAA, tested the effect of several naturally occurring bases on this decarboxylation, finding only adenine active at all. Although the conditions of the experiments reported herein are somewhat different from those of Breusch, the increase of 130% over the control obtained for adenine sulfate at 0.05 *M* agrees fairly well with the value of 20% increase for adenine sulfate at 0.005 *M* reported by that author. The effect of amino acids herein reported, in view of their normal concentration of from 20 to 40 milliequivalents/l. in the tissues (20), suggests that these extracts owed their catalytic activity to the amino acids they undoubtedly contained.

It has been demonstrated by Kornberg *et al.* (3) that various multivalent cations form more or less unstable complexes with OAA and other  $\beta$ -keto acids. A similar suggestion has been made for aniline by Wohl and Oesterlin (17) who postulated an anilide of OAA which broke down spontaneously to form carbon dioxide and the anilide of pyruvic acid. This anilide would then react with another molecule of OAA to form the anilide of OAA and a molecule of pyruvic acid. They prepared the anilide of pyruvic acid, but apparently did not test the hypothesis.

Langenbeck (21) suggested an intermediate Schiff-base formation in the catalytic effect of aniline on the decarboxylation of phenylglyoxylic acid. An activated complex between amino acid and OAA would be a likely pathway for the non-enzymatic decarboxylation of OAA as we have observed it, particularly since cations known to form complexes with OAA are partially inhibited in the presence of amino acids. An alternate explanation for the generally non-additive effect noted in Table II might be the phenomenon recently demonstrated by Neuberg and Mandl (22) whereby multivalent cations form complexes with amino acids.

In view of the relatively great effect of amino acids on the decarboxylation of OAA at concentrations approximating the total amino acids in tissues, and in view of the fact that these concentrations, and particularly the relative concentrations of specific amino acids (23), are subject to considerable fluctuation in both normal and pathologic states, the role of amino acids in the oxidative metabolism of carbohydrate would seem to bear investigation. An increased concentration of amino acids in the tissues might be expected to lower the available OAA sufficiently to allow the keto acids derived from amino acids to form carbohydrate. The reported rise in plasma amino acids upon administration of adrenocortical hormone (24) suggests a like rise in the tissues, which, in the light of the present report, might furnish some insight into the problem of gluconeogenesis.

### CONCLUSIONS AND SUMMARY

1. A catalytic effect of amino acids on the non-enzymatic decarboxylation of oxalacetic acid has been demonstrated.
2. At .05 *M* all amino acids tested increased decarboxylation of OAA from two to twenty-four fold.
3. This catalytic effect has been shown not to be due to contaminants in the amino acids.
4. The effect of multivalent cations has been shown to be, at most, additive, and usually the amino acid inhibits the effect of the cations.
5. The catalytic effect of amino acids occurs at concentrations approximating those found in tissues.
6. The catalytic effect of a particular amino acid is directly proportional to its concentration over the range 0.01-.05 *M*.
7. A possible role of this stimulation of the breakdown of OAA has been suggested.



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# The Activation by Heat of Triose Phosphate Dehydrogenase

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## INTRODUCTION

The rather unusual phenomenon of the activation by heat of a pure enzyme has been observed during the course of a study of the kinetics of the heat-inactivation of D-glyceraldehyde-3-phosphate dehydrogenase, the enzyme which catalyzes the oxidation of D-glyceraldehyde 3-phosphate to phosphoglyceric acid during carbohydrate breakdown.

It has been found that the heating at approximately 60°C. for short periods of time of a sample of enzyme which has partially inactivated spontaneously may, under certain circumstances, lead to an increase in activity rather than to the inactivation normally expected.

A study of the process has been made both because of its unique nature and because it was felt that further examination of the phenomenon would yield data of interest in the interpretation of the inactivation and denaturation of enzymes. A preliminary note describing earlier observations has already been published (1).

The reversal of the inactivation and denaturation of trypsin has been observed, and extensively studied, under certain conditions of temperature and pH (2). Similar effects have been found for serum albumin, hemoglobin, chymotrypsin, pepsinogen, and insulin.<sup>4</sup>

An extensive review of the literature, however, indicates that the reactivation of an enzyme by heat has rarely been observed before. Bertrand *et al.* (3) have described the heat activation of sucrase in yeast extracts but, largely because of the heterogeneous

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<sup>4</sup> An extensive list of references is given by NEURATH *et al.*, *Chem. Revs.* **34**, 243-57 (1944).

nature of the extracts, the results are somewhat dubious and do not readily lend themselves to any explanation.

During the course of this investigation a report has been published by Holstee (4) on a study of the heat activation of crystalline urease, an enzyme which, like triose phosphate dehydrogenase, depends for its catalytic activity on the presence of mercapto groups. It has been shown that the process is completely reversible; and an explanation of the phenomenon has been advanced which postulates that the reactivation consists of the dissociation by heat of urease molecules whose activity has been previously reduced by association.

Particular attention has been devoted in this study to the kinetic, rather than to the chemical, aspects of the problem. Factors of importance in inactivation and denaturation processes, such as pH, temperature coefficient, reversibility, etc. have been investigated.

### MATERIALS

*Triose phosphate dehydrogenase* (TPD) was prepared according to the method developed by Cori *et al.* (5) and recrystallized six times before use. Under ordinary conditions it was stored in 66%  $(\text{NH}_4)_2\text{SO}_4$  at 0°C.; but the greater part of the experiments described in this report were carried out with enzyme which had been dialyzed free from salt and which was then stored as a concentrated solution (approximately 10 mg./ml.) at 0°C.

*Coenzyme 1* (CoE 1). Most of the coenzyme used was prepared in this laboratory from Springer's yeast according to the method of Williamson and Green (6), as modified by Ochoa (7). Several samples prepared in the Bios and Schwartz laboratories were also employed. The purity was therefore not constant; but this was of secondary importance since control experiments were, in every instance, conducted on unheated samples of enzyme.

*D-Glyceraldehyde phosphate* (GAP) was prepared according to the method of Fischer and Baer (8) and was used as the dioxane-brominated compound.<sup>5</sup>

### EXPERIMENTAL PROCEDURE

The oxidation of GAP by triose phosphate dehydrogenase requires CoE 1 and phosphate; under these conditions it is reversible. The substitution of arsenate for the phosphate renders the reaction irreversible, and all measurements of activity described in this report have been made under these conditions. The method used was that originally introduced by Warburg and Christian (9) *i.e.*, measurement of the increase in height of the absorption band of reduced coenzyme at 340  $m\mu$  as a function of time in the 1 cm. cells of the Beckman spectrophotometer.

<sup>5</sup> We wish to acknowledge the assistance of Mlle B. Tchoubar in the preparation of this compound.

### *Composition of Cells*

The composition of the reaction cells was similar to that employed by Cori (5) and usually consisted of 0.03 *M* pyrophosphate buffer; 0.006 *M* sodium arsenate;  $0.5 \times 10^{-3}$  *M* glyceraldehyde phosphate; and  $0.34 \times 10^{-3}$  *M* coenzyme. The concentrations of CoE 1 and GAP were varied according to the conditions of the experiment. The pH of the reaction cell was maintained at 8.5–6 by means of the pyrophosphate buffer and the CoE 1 and GAP partially neutralized with alkaline pyrophosphate before use.

### *Activity Measurements*

Activities were usually measured by adding GAP to the other constituents of the reaction mixture and by reading optical densities at 30-sec. or 1-min. intervals in the spectrophotometer.

### *Calculation of Activity*

The initial velocity of the reaction was taken as a measure of enzyme activity and was calculated from the density readings as follows. Since all activity curves were of the same form, a cubic equation was fitted to the origin and the first three density readings at 30, 60, and 90 sec. or 1, 2, and 3 min. The slope at the origin was then obtained by simple differentiation. For density readings  $D_1$ ,  $D_2$ ,  $D_3$  at 30, 60, and 90 sec., respectively,

$$V_{\text{initial}} = 6D_1 - 3D_2 - 2/3D_3.$$

This method of calculation has been used more extensively in a study of the heat inactivation of this same enzyme (10) where it was shown that the rates, when calculated in this way, are proportional to the enzyme concentration.

### *Temperature Control*

Temperature control in the spectrophotometer cell compartment was maintained by circulating liquid from a constant-temperature bath through the circulating coil assembly of the spectrophotometer. In addition, the temperature in each reaction cell was measured, immediately following an activity measurement, by means of a small mercury thermometer; and, where necessary, corrections made for

variations in temperature by use of the known temperature coefficient of the reaction (11).

### *Method of Heating*

Since 0.2 ml. of enzyme was usually required for an activity measurement, 0.4 ml. was the volume usually heated. This was pipetted into a thin-walled glass test tube ( $8 \times 3/4$  cm.) which had been previously immersed in a constant temperature bath ( $\pm 0.1^\circ\text{C}$ ). Since accurate kinetic measurements were not usually involved in this work, no particular attention was paid to the time required (approximately 30 sec.) for the enzyme solution to reach the bath temperature which was usually within the range  $55\text{--}65^\circ\text{C}$ .

### *Preparation of Enzyme Samples*

Most of the enzyme samples were "aged" at  $0^\circ\text{C}$ . as dialyzed solutions. For any series of experiments an aliquot of the solution was removed, made up to the desired concentration<sup>6</sup> and pII with water and buffer, and then treated as desired. Some spontaneous inactivation at room temperature was frequently necessary before activation was observed, although activation was occasionally observed for samples just removed from the cold room.

For nondialyzed samples, the enzyme crystals were centrifuged down, the  $(\text{NH}_4)_2\text{SO}_4$  decanted, and the solution then made up as indicated above.

### *Coagulation*

Heated samples of enzyme frequently showed coagulation during both inactivation and activation. Since the coagulum increased the density readings, this might have been advanced as an "explanation" for the activation process. Two observations, however, completely eliminated this possibility.

It was first noted that the amount of coagulation in the cell reached a maximum immediately following addition of the enzyme and subsequent mixing, and did not change following this. This correction therefore entered as a constant factor during the density readings and did not affect the velocity measurements.

<sup>6</sup> Protein concentrations varied from 0.5 to 3.0 mg./ml. These concentrations are not stated explicitly since it was later found that it was not protein concentration which was of importance but the relationship of active to inactive protein in any sample, a relationship which was not known.

In addition, when completely inactivated enzyme solutions were heated, no activity was observed in the spectrophotometer, notwithstanding the fact that considerable coagulation may have taken place. As a result, the factor of coagulation was not considered further in the study of the activation process.

*Heat Inactivation* data for the enzyme are given in a separate report (10) which also contains a more complete description of the experimental technique.

## RESULTS

### *General Effects*

If a fresh solution of triose phosphate dehydrogenase is heated at 60°C., both in the presence or absence of  $(\text{NH}_4)_2\text{SO}_4$ , it will inactivate at a rate depending on the pH, enzyme concentration, etc. (10). However, if this enzyme solution is allowed to age, either at 0°C. or at room temperature, heating may then result in an increase in the activity of the enzyme.

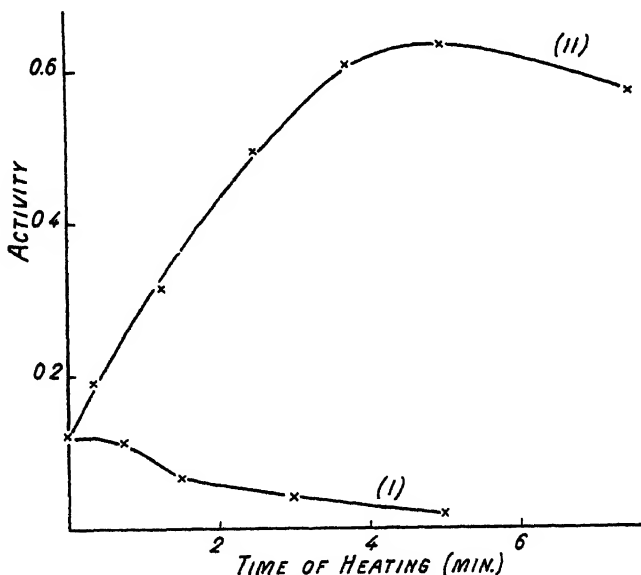


FIG. I. Activation in phosphate buffer at pH 5.6 at 60.0°C. (I) Behavior of fresh enzyme solution. (II) Activation of "aged" solution.

TABLE I

*Heat Activation of Triose Phosphate Dehydrogenase at 62.5°C.**"Aged" dialyzed enzyme, removed from cold room (0°C.) and allowed to decay at room temperature in 0.04 M phosphate buffer pH 5.6*

Inactivation (% original activity at room temperature)	39	47	57	93
Activation by heat (% increase)	11	16	16	530

Figure 1 illustrates the behavior of two similar solutions of different "age." The solution that shows activation (curve II) had been stored in crystalline form for about 4 weeks at 0°C., dialyzed to remove all traces of salt, and then stored again for about 4 weeks in the cold room. Curve I represents the behavior of a sample prepared from enzyme which had been stored for only 1-2 weeks in  $(\text{NH}_4)_2\text{SO}_4$ .

Taking the time of final recrystallization of the enzyme as zero time, activation could be observed when the enzyme was about 1-2 months old. During this period the activity will have diminished by more than 50%. The increase in activity upon heating has been found with four

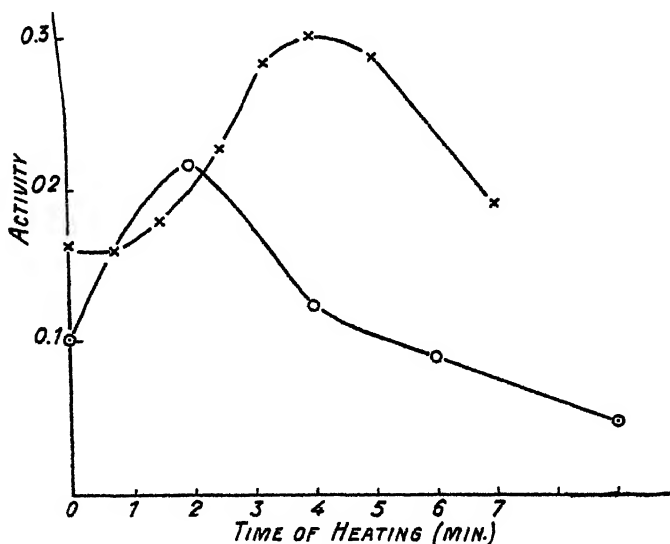


Fig. 2. Activation and inactivation of two sample TPD solutions at 60°C.

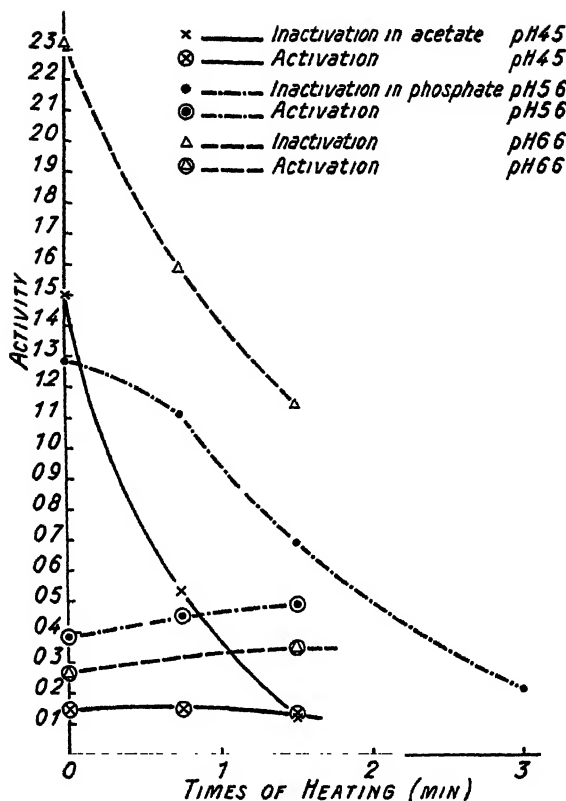


FIG 3 Activation of TPD as function of pH at 60°C

different enzyme preparations and depends on the age of the preparation, pH, temperature at which it is heated, and time of heating.

In general it has been found that the percentage activation with heat increases (a) with age and (b) with the extent of spontaneous inactivation of the enzyme. Although not essential, prolonged spontaneous inactivation at room temperature usually magnified the extent of heat activation. This inactivation that had taken place at room temperature could occasionally be completely reversed by heating the enzyme under suitable conditions. It has, however, never been possible to attain the activity originally shown by the freshly crystallized enzyme. Table I shows the effect of heating an enzyme solution at various stages of inactivation at room temperature. Very large percentage increases



could be obtained when the enzyme activity was very low; but it has not been possible to activate a preparation which had completely inactivated.

### *Heating Times*

These were necessarily quite short, for, if heating was unduly prolonged, inactivation commenced. Figure 2 illustrates the typical behavior with two different enzyme solutions. The time taken to reach maximum activity depends on the history of the particular sample and the temperature at which it is heated. At 60°C., for example, this time may vary between 1-7 min.

### *Effect of pH and Buffer*

The rate of inactivation of a fresh preparation of TPD as a function of pH and temperature has already been measured (10). In measuring the activation effect, the activities measured after definite heating periods have been compared with those normally obtaining with a fresh enzyme at that pH and temperature. The comparative results are illustrated in Fig. 3, which indicate that the activation effect, which is very small, is not dependent to any marked extent on the pH, at least in the range from pH 4.5-6.6. The different form of the inactivation curve at pH 5.6 may be due to some activation occurring in the first 45 sec.

The curves shown in Fig. 3 were obtained with acetate and phosphate buffers. Similar effects were observed in the presence of citrate buffer, arsenate ion, and in solutions dialyzed free from salt and unbuffered.

### *Reversibility*

It was found that a solution which had been activated at 60°C. would then start to inactivate at room temperature in a manner similar to that of a normal unheated sample. A series of experiments were then performed to determine whether such a sample could be again activated by heating.

A relatively large volume of enzyme was heated for successive periods of time until the activity had reached its maximum value, and then commenced to inactivate, as determined by continual sampling and measurements of activity. It was then allowed to stand at room temperature where the activity decreased over a period of a few hours. The solution was then again tested for possible heat activation.

Figure 4 illustrates the results for a typical experiment of this kind. The total heating times are plotted as abscissae, *i.e.*, the 6.5-min. heating time represents 5.5 min. preliminary heating plus 1 min. heating after inactivation at room temperature. The results indicate that the process is partially reversible.

#### *Temperature Coefficient*

Since activation was observed in the temperature range of 55–65°C., it seemed of interest to measure the temperature coefficient of the process. However, this type of measurement was inherently difficult since heat inactivation (with a large temperature coefficient) probably occurs simultaneously to some extent and this will tend to obscure any coefficient in the opposite direction.

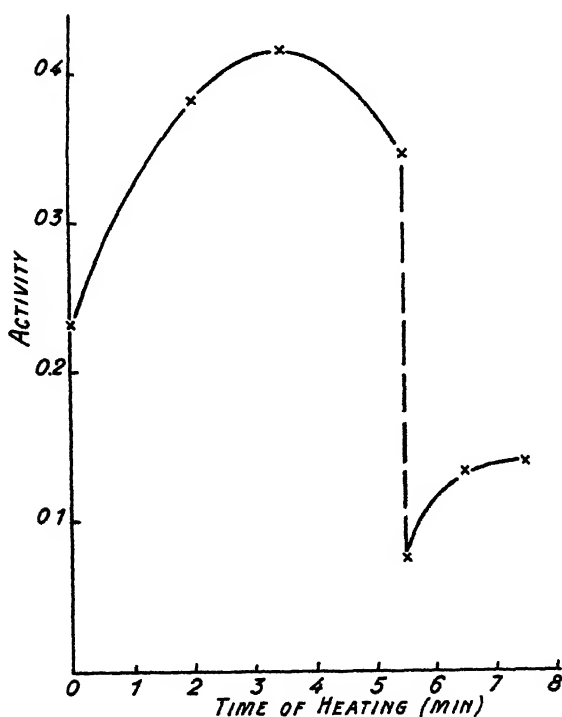


FIG. 4. Reversibility of activation process.

TABLE II

*Measurement of Temperature Coefficient of Activation Process*

Temperature	Time of heating	Activity	Activation
°C.	min	min <sup>-1</sup>	per cent
57.0	0.75	.122	15
	1.50	.177	
60.0	0.75	.145	51
	1.50	.223	

Table II shows the results of such an attempt and the result, while inconclusive, does indicate that some temperature coefficient does exist which is probably masked by the heat inactivation effect and is therefore not observed.

#### *Effect of Cysteine*

The activating influence of cysteine on the activity of TPD has been established as being due to the reduction of —S—S— groups to —SH, the activity of the enzyme being dependent on the latter groups (12). It appeared of interest, therefore, to examine whether, following heat activation, further activation could be obtained with cysteine.

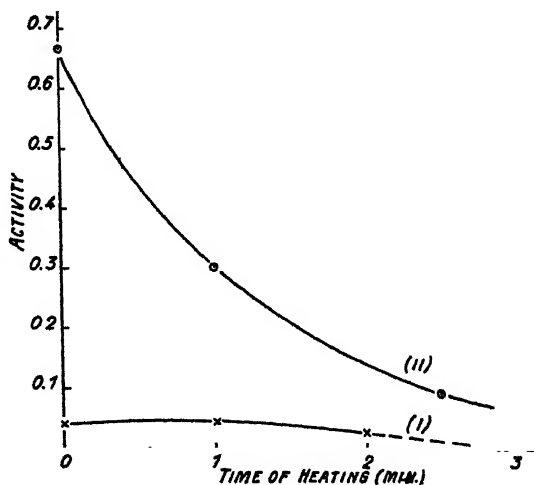


FIG. 5. Effect of cysteine activation. (I) Normal course of activation and inactivation. (II) Activity in presence of cysteine.

Samples of the enzyme were heated for various periods of time and the resulting activities measured in the absence of cysteine; curve I, Fig. 5, shows the general behavior, a little activation being observed. The procedure was then repeated with samples of the same TPD solution, but the activities in this case were measured after the heated samples of enzyme had been incubated for 5 min. in the cells in the presence of cysteine which had previously been neutralized. Although all samples show activation due to cysteine, the net result (curve II of Fig. 5) is a decrease in activity as a function of time of heating. This would appear to indicate that some of the S—S groups of the enzyme, which are potentially reducible by cysteine, are inactivated or modified by heating; and this effect completely overshadows any activation resulting from heating.

#### *Effect of Inactive Enzyme*

All samples of enzyme for which activation was observed contained a certain amount of inactive enzyme, the proportion of which, to active enzyme, increased as the activity decreased. The relationship of age of the solution to the activation attainable led naturally to the investigation of the role, if any, played by inactive protein in the activation process.

Figure 6 shows the effect of the addition of a large amount of inactive protein to an active enzyme solution. Curve I gives the normal behavior of the active enzyme sample. Curve II illustrates the behavior in the presence of an added quantity of inactive protein, the concentration of active enzyme per unit volume being the same in both cases. Heating of the inactive enzyme itself resulted in no change. The activation resulting from the addition of inactive protein is very striking and was repeated a number of times. It certainly confirms the supposition that the inactive protein plays more than a passive role in the heat activation process.

#### *Effect of Arsenate*

Because of the fact that arsenate participates in the oxidation reactions catalyzed by TPD, the possibility of heat activation in its presence was also looked into; and, as pointed out above, the results were positive. In view of subsequent findings that activation occurred in the presence of other ions, this in itself would appear to be of secondary importance.

In sharp contrast, however, was the behavior of TPD solutions in the presence of arsenate at room temperature. Contrary to the expected spontaneous inactivation, the activity remained constant for a day or two and then started to increase (curve I of Fig. 7). This increase in activity continued until the activity was as much as three times or more than that observed when the solution was initially prepared

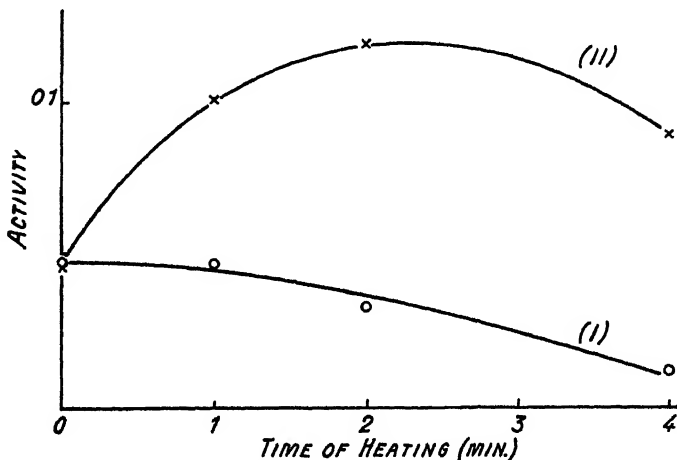


FIG. 6. Effect of inactive protein on activation. (I) Inactivation of normal TPD solution. (II) Activation in presence of excess inactive protein.

Furthermore, heating at a given time produced further activation, as shown by curve II. Particularly noteworthy is the fact that the percentage heat activation increased with the age of the solution as for samples which inactivated spontaneously before activating.

### *Effect of Ultraviolet Light*

Fresh solutions of triose phosphate dehydrogenase irradiated with ultraviolet light will inactivate in a manner, similar in some respects only, to heat inactivation (13). The possibilities of activation by ultraviolet light were therefore investigated with a view to elucidating any similarities in the two processes.

Some authors have reported findings indicating activation of enzymes by ultraviolet light, but examination of the original data shows little, if any, foundation for these reports.

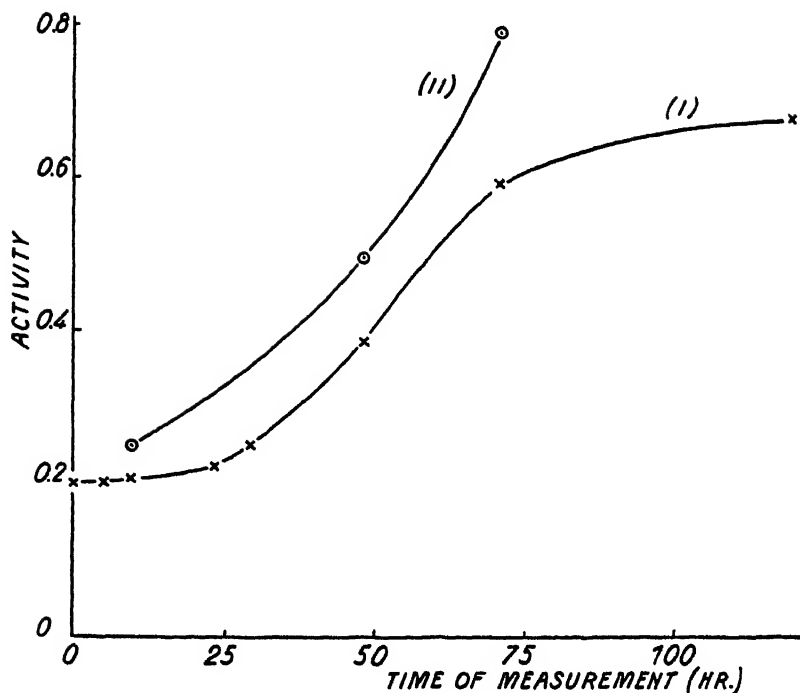


FIG. 7. Activation in presence of arsenate ion. (I) Activity of sample maintained at room temperature. (II) Activity after heating at 60°C.

All attempts to activate TPD with ultraviolet light proved unsuccessful. Attempts at activation were made using different samples of enzyme and, in particular, several samples which were known to show heat activation.

While these results may not completely rule out the possibility of activation by ultraviolet light, the negative finding may have theoretical significance (see discussion below).

#### *Effect of Cold Temperatures*

In view of the findings of Nord and co-workers (14) that the activity of solutions of zymase was increased by first freezing to  $-80^{\circ}\text{C}.$ , similar experiments were attempted with TPD. No evidence of activation was found; and, in fact, one sample frozen to  $-180^{\circ}\text{C}.$  showed more inactivation upon thawing than a similar control sample

kept at room temperature. However, no determinations of the size of particles of the TPD solutions were carried out either before or after freezing.

### *Bacterial Contamination*

Since no special precautions were taken to maintain sterile conditions during this investigation, the possibility of bacterial contamination playing some role in the activation process was considered.

However, the activation process was observed in experiments conducted under sterile conditions. As a control for these experiments, bacterial counts were made on solutions which showed activation; negligible contamination was observed. It is, therefore, considered unnecessary to outline in detail this aspect of the work.

### DISCUSSION

In previous reports of the reversal of the inactivation and denaturation of enzymes and proteins, the protein was denatured by some agent such as heat, acid, *etc.*; and, insofar as it was not maintained in the presence of the denaturing agent for too long a period, the process could be reversed by removal of the specific agent responsible for the inactivation.

The results described in this report represent, in some respects, a reversal of inactivation; but the characteristics of this system are quite unlike those previously described inasmuch as the reversal is obtained by heating the enzyme in the range of temperatures which normally lead to inactivation and denaturation.

As far as can be ascertained Hofstee's results with crystalline urease (4) represent the only other instance of heat activation similar to the phenomenon found for triose phosphate dehydrogenase. However, with the exception of the range of temperatures used (60°C. only) and the times of heating (5 min.), the urease activation exhibits a number of marked dissimilarities from that for TPD.

(1) The urease inactivation appears to be completely reversible.

(2) The urease could be activated immediately after preparation. Neither of these phenomena were reproducible with TPD. Hofstee explains the urease activation on the hypothesis that some association of the molecules results in the masking of a portion of the active groups with resulting drop in activity; heating then results in a "dissociation" of the molecules, with consequent unmasking of active groups and

therefore an increase in activity. An examination of Sumner's data for sedimentation of urease in the ultracentrifuge (15) does in fact indicate the presence of two components with higher molecular weights. Hofstee is able to explain most of the phenomena observed with urease on the basis of this hypothesis.

Because of the different behavior exhibited by TPD heat activation, it seems necessary to seek another explanation for the mechanism involved.

Some clues as to the nature of this mechanism are offered by the heat activation of a sample of active enzyme upon the addition of completely inactive protein (Fig. 6). Apparently some intermolecular process is involved. It is well known that heating mercapto enzymes results in the unblocking of some of these groups, so that they can then be readily detected by means of the nitroprusside test (16). It seems reasonable, therefore, to assume that some of the  $-SH$  groups liberated from the inactive protein "reduce" the  $S-S$  groups of the active molecules resulting in a net increase of activity. Aging of the enzyme solution is analogous to the addition of inactive protein.

This "reducing" effect, while similar in some respects to that effected with cysteine, may not necessarily be the same (see below). The activating effect of heat does increase with decreasing concentration of active enzyme, as does the reducing effect of cysteine.

This hypothesis, however, is certainly not applicable to the results obtained by incubation of heat-activated enzyme in cysteine, which show that the  $S-S$  groups potentially reducible by cysteine are destroyed by heating. It is, however, quite possible that there may be two types of groups in the triose phosphate dehydrogenase molecule: one is readily destroyed by heat; while the other, that reducible by protein  $-SH$  groups, is more resistant to the action of heat. While no direct experimental evidence exists for these assumptions other than the results indicated above, the conception of the existence of  $-SH$  groups with different properties in a single enzyme is not new, and has been postulated by a number of observers for urease (17).

With regard to the proposal of reduction of  $-SH$  groups by interaction between proteins containing such groups, an interesting comparison is offered by the results of Kather (18) who found that ultra-violet irradiated egg-white inactivates insulin. The phenomenon is explained on the supposition that photochemically liberated  $-SH$



groups in the ovalbumin reduce the active S—S groups in insulin to the inactive —SH form.

The nonexistence of activation by ultraviolet light finds a ready explanation in the proposals of Mirsky and Pauling (19). It is suggested that, in place of the breaking of many hydrogen side-bonds by heat-inactivation, ultraviolet inactivation consists in the breaking of one of the bonds in the main chain of the molecule. Under these conditions, fewer side chains would be affected and fewer —SH groups released, with the consequent absence of any activating effect.

During the course of these experiments a series of long-term stability studies were carried out on TPD solutions at various pH's and at two temperatures, 0° and 30°C. Although the results were not always reproducible and in many cases not conclusive, a few points merit attention. Many samples showed remarkable stability at 30°C. for as long as a month; several exhibited spontaneous increases in activity of 30% or so. Frequently, too, the stability of the samples at 30°C. was much greater than for exactly similar samples at 0°C. The results obtained for TPD solutions in the presence of arsenate are particularly striking (Fig. 7). The steady spontaneous increase in activity over a period of 2 weeks by as much as 300% was found to be readily reproducible a number of times. While these observations will undoubtedly find an explanation in terms of intermolecular oxidation or reduction of —SH and S—S groups, it is felt that further work is required for the elaboration of a theory which will explain all the facts.

It would be of interest to study other —SH-containing proteins under similar conditions both for activating effect on triose phosphate dehydrogenase and for self-activating effect.

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#### SUMMARY

1. The enzyme triose phosphate dehydrogenase may be reactivated by heating in the range 55.0–65.0°C. under certain conditions.

2. This effect has been studied as a function of the history of the enzyme solution, in particular the time of storage at different temperatures.

3. The influence of pH, temperature, cysteine, and the presence of inactive enzyme on the phenomenon has been studied.

4. In the presence of arsenate, the enzyme undergoes slow spontaneous reactivation.

5. The hypothesis that the activation is a result of the interaction between mercapto groups of the inactive protein and disulfide groups of the active protein has been advanced.

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# Growth-Promoting Activity of Vitamin B<sub>12</sub> in Mice Fed Massive Doses of Atabrine

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## INTRODUCTION

It is becoming increasingly apparent that the effects obtained in an experimental animal following administration of drugs or related products are dependent on the nutritional state and the diets employed. In acute deficiencies of essential nutrients it is readily recognized that resulting abnormalities in cellular metabolism may profoundly affect response to certain drugs. What is less well recognized, however, is that these drugs themselves may precipitate a deficiency state (1). Thyroid administration, for example, may induce a deficiency of thiamine, pyridoxine, and pantothenic acid (2), while dicumarol, salicylates, and other drugs may precipitate a deficiency of vitamin K (3-5). Popper *et al.* (6) have recently observed that vitamin B<sub>12</sub> counteracted in part the effects of CCl<sub>4</sub> intoxication in the rat. In the present communication data are presented concerning the growth-promoting effect of vitamin B<sub>12</sub> in the immature atabrine-fed mouse.

## EXPERIMENTAL

The basal ration employed in the present experiment consisted of dextrin<sup>1</sup> 56%, casein<sup>2</sup> 25%, soybean oil 12%, salt mixture<sup>3</sup> 5%, and cellulose<sup>4</sup> 2%. To each kilogram of the above diet were added the following synthetic vitamins: thiamine hydrochloride 20 mg., riboflavin 20 mg., pyridoxine hydrochloride 20 mg., nicotinic acid 60 mg., calcium pantothenate 60 mg., biotin 4 mg., folic acid 10 mg., *p*-aminobenzoic acid 400 mg., inositol 800 mg., 2-methyl-naphthoquinone 10 mg., and choline chloride

<sup>1</sup> White Dextrin Merck, N.F.V., Merck and Co., Inc., Rahway, New Jersey.

<sup>2</sup> Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>3</sup> Sure's Salt Mixture No. 1 (7).

<sup>4</sup> Ruffex, Fisher Scientific Co., St. Louis, Mo.

2 g. Each mouse also received once weekly a vitamin A-D concentrate<sup>5</sup> containing 25 U.S.P. units of vitamin A and 2.5 U.S.P. units of vitamin D together with 1.5 mg. of  $\alpha$ -tocopherol acetate.

Seventy-five male mice of the CC-1 strain<sup>6</sup> were selected at 23–25 days of age and an average weight of 12.2 g. for the following experiment. Animals were placed in metal cages with raised screen bottoms to prevent access to feces and were fed the following diets *ad lib.*: (a) basal ration alone; (b) basal ration plus atabrine;<sup>7</sup> (c) basal ration plus atabrine plus the following vitamin supplements/kg. of diet: thiamine hydrochloride 20 mg., riboflavin 20 mg., pyridoxine hydrochloride 20 mg., nicotinic acid 60 mg., calcium pantothenate 60 mg., biotin 4 mg., folic acid 10 mg., *p*-aminobenzoic acid 400 mg., inositol 800 mg., and 2-methyl-naphthoquinone 10 mg.; (d) basal ration plus atabrine plus 30  $\mu$ g. vitamin B<sub>12</sub><sup>8</sup>/kg. of diet; and (e) basal ration plus atabrine plus 10% Extracted Liver Residue.<sup>9</sup> Previous findings (8) indicate that this liver fraction, consisting of the coagulated, water-insoluble material remaining after removal of the extractable water-soluble substances, is a potent source of a growth-promoting factor for the immature atabrine-fed rat. Atabrine was incorporated in the above diets at a level of 500 mg./kg. of diet. Supplements were added to the basal diet in place of an equal amount of dextrin. Feeding was continued for 15 days (15 mice per group).

The findings are summarized in Table I. Data were computed on the basis of the top 12 animals in each group to minimize variations in averages due to early deaths, infection, and atypical responses on the part of individual mice. A marked retardation in growth was observed in immature mice fed the basal diet supplemented with massive doses of atabrine. Gain in body weight was significantly increased by supplements of vitamin B<sub>12</sub> or Extracted Liver Residue. Doubling the B-vitamin content of the basal diet, however, was without significant effect. Although both vitamin B<sub>12</sub> and Extracted Liver Residue promoted growth in immature mice fed massive doses of atabrine, gain in body weight was most marked on the liver-residue diet. Animals

<sup>5</sup> Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D/g.

<sup>6</sup> Animals were obtained from a local breeder. The CC-1 strain was derived from crossbreeding the Webster and ABC strain.

<sup>7</sup> Atabrine (Quinacrine-HCl powder), Winthrop Chemical Co., New York.

<sup>8</sup> Cobione (Crystalline Vitamin B<sub>12</sub> Merck), Merck and Co., Rahway, New Jersey.

<sup>9</sup> Extracted Liver Residue, Wilson Laboratories, Chicago, Ill.

fed on the latter ration did not differ significantly in weight from controls on an atabrine-free diet. With the exception of growth no significant differences were observed grossly on any of the diets employed.

Previous findings indicate that Extracted Liver Residue contains one or more factors apparently distinct from any of the known nutrients (including vitamin B<sub>12</sub>) which is required in increased amounts by immature rats fed massive doses of desiccated thyroid (9-11), thyroxin (12), thyroglobulin (12), iodinated casein (12), atabrine (8), and

TABLE I

*Effects of Vitamin B<sub>12</sub> and Extracted Liver Residue on the Gain in Body Weight of Immature Mice Fed Massive Doses of Atabrine*

Dietary supplement	Atabrine dosage	No. of mice	Initial body wt.	Gain in body wt. 15-day period <sup>a</sup>
	<i>mg./kg. of diet</i>		<i>g.</i>	<i>g</i>
None	0	12	12.4	10.8±0.5
None	500	12	12.3	4.6±0.3
B Complex vitamin mixture (without vitamin B <sub>12</sub> )	500	12	12.6	5.3±0.3
Vitamin B <sub>12</sub>	500	12	11.9	7.2±0.4
Extracted Liver Residue	500	12	12.5	10.3±1.1

<sup>a</sup> Including standard error of the mean calculated as follows:  $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

promin (13). Extracted Liver Residue has similarly been found to promote growth and prolong survival in immature mice fed massive doses of thyroid, thyroxin, and iodinated casein (14).

### SUMMARY

In the present experiment massive doses of atabrine caused a marked retardation in the growth of immature mice which was *partially* counteracted by the administration of crystalline vitamin B<sub>12</sub>. Extracted Liver Residue, however, restored growth to virtually normal levels. Since the dose of vitamin B<sub>12</sub> employed was considerably in excess of minimum requirements, it appears that Extracted Liver Residue may contain one or more factors other than vitamin B<sub>12</sub> which

contributed in part to its growth-promoting effect under conditions of the present experiment. Present findings suggest that this factor is distinct from any of the known B vitamins.

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# Relations Between the Rate of Renewal and the Intracellular Localization of Ribonucleic Acid

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## INTRODUCTION

While thymonucleic acid is found localized in the chromosomes, ribonucleic acid (RNA) is present at the same time in the cytoplasm, the nucleolus, and, most likely, in the chromosomes themselves (1,2). Within the cytoplasm it appears to be one of the constituents of lipo-protein particles having rather varied composition and speeds of sedimentation; it may also exist in the "free" form, incapable of sedimentation in 1 hr. at  $100,000 \times g$  (3,4,5,6).

The question may be posed as to whether its presence at certain defined points of the cell is related to the role it may play in the functioning of mechanisms which are themselves localized. The apparent relation between RNA and the synthesis of proteins has already led to numerous hypotheses on this subject, such as the idea that the nucleus is the seat of synthesis of histones (2), that the cytoplasm particles play a role in the synthesis of proteins (4), and are the geneticists' plasmagenes (7). Finally, considerations of an entirely different nature have recently led Gale (8) to the unexpected and suggestive discovery that the superficial ribonucleoprotein of gram-positive bacteria appears to be indispensable for the accumulation within the cell of certain amino acids present in the nutritive medium.

All these hypotheses are at present somewhat speculative, a fact not astonishing in view of our ignorance of the reactions in which RNA participates within the cell. We have attempted to obtain some information in this obscure field of biochemistry by ascertaining if there exists some marked difference in the rate at which the replacement of phosphate groups of RNA molecules, by "marked" inorganic phos-

phate, takes place, according to the type of cellular particle they form part of.

The appearance of "marked" phosphate groups in molecules of RNA can be explained in a variety of ways, and such a study by itself would not reveal very much regarding the role of the RNA of the nucleus or the cytoplasmic granules. However, should RNA play a particularly important role in the chemical activity of certain particles, its higher "turnover" rate within these particles would draw our attention to them and would lead us to attempt to interpret the information thus obtained by comparison with that obtainable by other methods.

On the other hand, it should be noted that the existence of appreciable differences in the "turnover" rates of the RNA of various fractions of cytoplasmic particles, separable by centrifugation of tissue extracts, would definitely prove that RNA is a constituent of these particles in the normal cell.

#### EXPERIMENTAL

Materials utilized were the liver of adult rats fasted for one night; the embryos of mice removed several days prior to parturition; and the cells of a colorless flagellate, *Polytomella coeca*, obtained by mass culture, and used during the slowing down of its period of multiplication which follows immediately after the exponential phase.<sup>1</sup> In all the experiments, sodium orthophosphate, marked with P<sup>32</sup>, was injected subcutaneously (rats, pregnant mice) or introduced into the culture medium (*Polytomella*) 2 hr. prior to the sampling and fractionation of the cells. The homogenization of these was effected at 1°C. with the aid of an "Osterizer" apparatus, in 10 volumes of 0.005 M phosphate buffer at pH 7.2. The duration was of 1 or 2 min. according to the material used. In several cases, homogenization was obtained in 30% sucrose.

##### *a. Fractionation of the Cytoplasmic RNA*

After the cellular nuclei had been eliminated by centrifugation for 20 min., at 2,100 or 3,000 r. p. m., as the case might be, the extract underwent a variable number of successive centrifugations at increasing speeds. Following each centrifugation, the pellet, resulting from the aggregation of a portion of the particles at the bottom of the tubes, was separated. The supernatant liquor of the last centrifugation was saved. The various pellets obtained were washed with buffer under the conditions of centrifugation in which they were initially formed. As has been shown by Chantrenne (6), we are concerned with arbitrarily selected fractions composed of particles the speeds of sedimentation of which are statistically grouped around a mean speed which characterizes each fraction. The precise conditions under which the fractionation was effected were those described by Chantrenne (6).

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<sup>1</sup> We are indebted for this material, which is excellent for cytochemical studies, to Dr. A. Lwoff, to whom we express our appreciation.



### *b. Isolation of the Nuclei of Rat Liver*

The initial method of Stoneburg (9), which is much more rapid and simpler than more recent methods, was used. For our purpose it was essential that the isolated nuclei be free from all traces of cytoplasmic RNA. The method of controlling the elimination of all possible fragments of cytoplasm was that of coloration on smears by the methyl green-pyronine mixture (Brachet).

### *c. Determination of the Specific Radioactivity of the P of RNA*

During the initial part of our researches (fractionation of the cytoplasmic RNA of mice embryos), the technique utilized for the isolation of the ribonucleotides of RNA was that of Schmidt and Thannhauser as modified by Juni *et al.* (10) and by ourselves (11). The main difficulty we encountered in using the method of Juni *et al.* was the inability to separate sufficiently well the ribonucleotides of RNA from the phosphoprotein or inorganic phosphate. We believe we have adapted it to our needs by rendering more complete the precipitation of phosphorus (free inorganic or detached from phosphoproteins), by repeated additions of non-labeled phosphate to the solution of ribonucleotides and its precipitation in the form of  $\text{MgNH}_4\text{PO}_4$ . Despite these supplementary precautions the specific radioactivity of the P of RNA, as measured by the method of Hammarsten and Hevesy (12), was always 35–45% less than that obtained by the modified method of Juni *et al.*, and a certain degree of uncertainty therefore attaches to our first results.

We believe we have considerably improved our technique by basing it on the following facts:

1. It is possible to reduce (by at least 20 times) the quantity of inorganic P of high specific radioactivity present in the solution of ribonucleotides obtained by the method of Juni *et al.*, by preventing its adsorption on the initial precipitate of proteins obtained by means of trichloroacetic acid. This result is achieved either by the addition of concentrated  $\text{Na}_2\text{HPO}_4$  to the tissue extract prior to precipitation by trichloroacetic acid, or by repeated washing of the precipitate with  $\frac{1}{4}$ -saturated  $\text{Na}_2\text{HPO}_4$ .

2. Following washing out of the excess phosphate, and delipidation with boiling alcohol-ether, the precipitate is treated as follows:

- (a) (if thymonucleic acid is present), with dilute caustic soda which liberates the ribonucleotides, the thymonucleic acid being subsequently precipitated at low pH (technique of Juni *et al.*);

- (b) (if it was possible previously to eliminate the cellular nuclei), with 5% trichloroacetic acid (100°, 15 min.) which frees the ribo-

nucleotides together with a variety of foreign substances much smaller than result by Juni's technique.

3. The solutions of ribonucleotides thus obtained contain (as impurities which interfere particularly with the measurements of the specific radioactivity of the P of RNA) inorganic P originating in the phosphoproteins or insufficiently eliminated following initial treatment with concentrated phosphate. The thorough elimination of inorganic P may be achieved by paper chromatography. The nucleotide solutions are led directly on to the paper and dried. An ascending chromatogram is obtained with the aid of water-saturated phenol. A solution of non-labeled nucleotides to which labeled inorganic P has been added is used for the preparation of a control chromatogram on which the position occupied by the inorganic phosphate is indicated by means of a Geiger counter, while that occupied by the nucleotides is determined either by the formation on the paper of brown uranyl ferrocyanide (13) or by cutting the paper into strips from which the nucleotides are eluted and then dosed by the orcinol method of Barrenscheen and Peeham (14). It was shown in this way that about 70% of the ribonucleotides in question are found in an area of the chromatogram well separated from that occupied by the inorganic phosphate, the displacement of which is small. A similar experiment, carried out by introducing sodium metaphosphate or pyrophosphate into the chromatographed nucleotide solutions, indicated that they separate out from the nucleotides just as readily as the orthophosphate.

The solution obtained by elution of the area occupied by the nucleotides and completely free from inorganic P was dried, and the radioactivity of the P of the RNA determined by means of a counter the background of which did not exceed 2-3 counts/min. (15). The powder, on which the determination of radioactivity was made, was ashed with the aid of perchloric acid, and the P determined colorimetrically by the method of Berenblum and Chain (16).

It should be noted that the chromatographic method is equally suitable for the separation of ribonucleotides from thymonucleic acid remaining polymerized; the displacement of the latter is less rapid.

The technique thus established has been applied to the study of RNA fractions of (a) the liver of the rat and (b) the cells of *Polytomella*.

## RESULTS

*a Comparison of the Specific Radioactivities of the Various Fractions of Cytoplasmic RNA<sup>2</sup>*

The fractionation of the RNA of the cytoplasm of the liver shows (Table I) that the specific radioactivity acquired in 2 hr. by the P of the RNA goes on decreasing rapidly from the fraction with the smallest speed of sedimentation (supernatant liquor,  $6 \times 10^4 \times g$ , 60 min.) to that where it is the greatest (pellet,  $1.3 \times 10^4 \times g$ , 10 min.). It should be noted that this distribution of the specific radioactivity of the cytoplasmic RNA has been observed in adult animals fasted during one night and where the hepatic cells were not in a phase of their activity allowing the accumulation of supplementary quantities of proteins and nucleic acids. We have proved that the determination of the specific radioactivity of the P of the total RNA of the liver, by the method of Hammarsten and Hevesy (12), gave results agreeing very well with those obtained by the chromatographic technique.

TABLE I  
*Fractionation of the Cytoplasm of Hepatic Cells*

	Pellet, $1.3 \times 10^4 \times g$ , 10 min			Pellet, $6 \times 10^4 \times g$ , 60 min			Supernatant $6 \times 10^4 \times g$ , 60 min		
	Tot act	P of RNA in $\mu g$	Spec act	Tot act	P of RNA in $\mu g$	Spec act	Tot act	P of RNA in $\mu g$	Spec act
1	30	85.2	35	52	33.3	156	16	7.6	210
2	69	59.0	117	120	57.2	210	212	24.6	863
3	15.5	92.5	17	35	116.5	30	43.5	29.5	147

The fractionation of the cytoplasmic extract of mice embryos (Table II) gave results differing in some respects from those obtained in the case of the liver of the rat. The fraction which remains in solution following centrifugation for 1 hr. at  $6 \times 10^4 \times g$  shows, as in the case of the liver, the highest specific radioactivity. Again, as in the case of the liver, the fraction with the intermediate speed of sedimentation (pellet,  $6 \times 10^4 \times g$ , 60 min.) shows a specific radioactivity 3 to 6 times

<sup>2</sup> In the tables following we shall indicate in arbitrary units (counts/min.) the activities observed on the totality of our preparations (tot. act.) and the activities referred to 100  $\mu g$ . of P (spec. act.).

TABLE II  
*Fractionation of the Cytoplasm of Mouse Embryos*

	Pellet, $1.3 \times 10^4 \times g$ , 10 min.			Pellet, $6 \times 10^4 \times g$ 60 min.			Supernatant, $6 \times 10^4 \times g$ , 60 min.		
	Tot. act.	P of RNA in $\mu g.$	Spec. act.	Tot. act.	P of RNA in $\mu g.$	Spec. act.	Tot. act.	P of RNA in $\mu g.$	Spec. act.
1	208	20.8	1,000	62	14.1	440	549	40.2	1,370
2	136	27.2	500	59	26.4	224	337	56	602
3	25	32.3	77	3	19.0	16	51	51	100

lower. On the other hand, the fraction with the highest sedimentation speed (pellet,  $1.3 \times 10^4 \times g$ , 10 min.), instead of possessing, as in the case of the liver, the weakest specific radioactivity, shows an activity practically equal to that of the fraction with the smallest speed. It would be interesting to examine other materials to see if this pronounced activity of the RNA of the largest particles (mitochondria?) is characteristic of tissues in active growth.

Up to the present, we have been unable to obtain complementary information on this subject, except by the study of *Polytomella coeca*, during growth. The results provided by fractionation are listed in Table III. The RNA distributes itself between the various fractions of the extract as in organs in growth or in the embryos of vertebrae; that is to say, an important portion of the RNA is not sedimented in 1 hr. at  $6 \times 10^4 \times g$ , and the higher the speed of sedimentation of these particles the lower is their content of this substance. The specific radioactivity is highest in the supernatant liquor obtained in 1 hr. at  $6 \times 10^4 \times g$ ; it is progressively lower in the fractions with higher

TABLE III  
*Fractionation of the Cells of Polytomella coeca*

	Tot. act.	P of RNA in $\mu g.$	Spec. act.
Pellet, 3,000 r. p. m., 10 min.	959	7.2	13,300
Pellet, 12,000 r. p. m., 10 min.	3,929	42.6	9,200
Pellet, 40,000 r. p. m., 10 min.	577	10.8	5,300
Pellet, 40,000 r. p. m., 60 min.	4,150	71.8	5,800
Supernatant, 40,000 r. p. m., 60 min.	1,355	13.0	10,400

speeds of sedimentation (up to the fraction separated in 10 min. at  $6 \times 10^4 \times g$ ), only to rise again up to the fraction with the highest speed of sedimentation (separated in 10 min. at 3,000 r. p. m.). At first sight, the results provided by *Polytomella* appear to confirm those obtained by the study of extracts of mice embryos. However a certain amount of caution is necessary here. In the extracts of *Polytomella* it has not been possible to recover the cellular nuclei, which are very small and difficult to characterize because of their extraordinarily low content of thymonucleic acid. It is therefore quite possible that one of our fractions may contain the nuclei, or fragments of them, and, consequently, RNA which may not be of cytoplasmic origin.

In order that the results just outlined may have some significance, we ought to be certain that no appreciable modification of the activity of the various fractions of RNA was produced during the frequent operations necessary for their separation (approximate duration, 6 hr.). Notwithstanding that all these operations were carried out at low temperature, it can by no means be excluded that appreciable enzymatic processes took place in the extracts. In order to determine the importance of such a source of error, measurements were made, at regular intervals, of the specific radioactivity of the P of the RNA from the total extracts of liver of mice embryos to which had been added labeled phosphorus following dispersion of the tissues. The activities observed barely exceeded those arising from sources of error, such as the adsorption of inorganic phosphate on the protein precipitates. If the "turnover" of the P of RNA takes place in the extracts, it is in any event 300 times too small to explain the differences recorded between the specific radioactivities attained by the various fractions of cytoplasmic RNA.

*b. Comparison Between the Specific Radioactivities of the Nuclear and Cytoplasmic RNA of Rat Liver*

Three types of experiment were carried out:

1. The livers of 2 rats, removed 2 hr. following injection of labeled phosphorus, are each divided into 2 portions, one of which is used for the isolation of the nuclei by the method of Stoneburg (9), the other for the preparation of a tissue extract in 0.005 M buffer at pH 7.4. This extract is freed of the nuclei by centrifugation at low speed as previously described. The isolated nuclei and the extract served as the

starting points for isolation of the nucleotides of the RNA by chromatography.

The observed radioactivities are shown in Table IV (A). They indicate that the nuclear RNA attained a specific radioactivity 10 times higher than that of the cytoplasmic RNA. The difference between these two radioactivities is probably much greater. In effect, centrifugation of the liver extract at low speed eliminates, besides the nuclei, an important portion of the largest cytoplasmic particles, the RNA content of which we know exhibits a specific radioactivity much feebler than that of other fractions.

TABLE IV  
*Nuclei and Cytoplasm of Hepatic Cells*

		Tot. act.	P of RNA in $\mu$ g.	Spec. act.
A	Nuclei	27.3	3.9	700
	Cytoplasm (supern. 3,000 r. p. m., 10 min.)	14.4	20.5	70
B	Nuclei	198.0	88.7	223
	Cytoplasm (supern. 40,000 r. p. m., 60 min.)	37.5	86.3	43

2. In a second experiment, we sought to compare the specific radioactivity of the RNA of the nuclei with that of the RNA of the cytoplasmic fraction exhibiting the strongest specific radioactivity, *i.e.*, the fraction which does not sediment in 60 min. at  $6 \times 10^4 \times g$ . The test was made on four rat livers the fragments of which were utilized either for the isolation of the nuclei or for the preparation of the extract by ultracentrifugation. As shown in Table IV (B), the radioactivity of the nuclear RNA is 5 times greater than that of the cytoplasmic RNA of the most radioactive fraction. Recalling that this cytoplasmic fraction is itself 7 times more radioactive, on the average, than the heaviest fraction, we conclude that, within a given cell, the radioactivity varies from 1 to 35 according to the fraction under consideration.

3. Finally, in a third experiment, the RNA of the isolated nuclei was compared with the RNA of the whole hepatic tissue. This experiment confirms the two preceding ones, the ratio of the two specific

TABLE V  
*Nuclei and Total Extract of Hepatic Cells*

	Tot. act	P of RNA in $\mu$ g	Spec act
Nuclei	24.3	7.0	347
	36.8	13.2	277
Total extract	52.9	68.4	77
	76.0	97.3	78

radioactivities having, as could be predicted, a smaller value than the ratio found in Exp. No. 1 (see Table V).

Since the publication by one of us (17) of a preliminary note on the specific radioactivity of various fractions of the RNA of embryos and organs in growth, several related studies have been published or have come to our attention.

Barnum and Huseby (18) appear to have carried out experiments most resembling ours. Their results are unfortunately known to us only through a short preliminary note which omitted description of the techniques used by them in the separation of the fractions and in the determination of the specific radioactivity of the RNA. Insofar as we can judge, the results agree with our own with the exception that no difference between the specific radioactivities of the microsomes and mitochondria was observed by them.

On the other hand, Marshak (19) reports that the specific radioactivity of the RNA of the nuclei is much greater than that of the RNA of the cytoplasm. His experimental techniques are, however, by no means convincing.

Finally, Hammarsten and collaborators (20) have found much larger quantities of  $N^{15}$  in the guanine and adenine of nuclear RNA than in the same compounds isolated from the cytoplasmic RNA of the liver of rats injected with labeled glycine. The differences observed are much smaller than those obtained by us in the case of rats injected with labeled phosphate. The explanation for this discrepancy may be that the time interval between the injection of the labeled substance and the killing of the animal was much longer in Hammarsten's experiments than in our own. In addition we do not know if the "turn-over" rate of the phosphate and of the purines of the nucleic acid is the same.

## DISCUSSION AND CONCLUSIONS

It is of some consequence to distinguish clearly the established conclusions which may be drawn from the preceding results, and the hypotheses or provisional interpretations to which they lead.

Since the discovery by Claude (3) of the cytoplasmic particles of embryos and tumors and the demonstration of their presence in the extracts of the most varied cells of mammals, invertebrates, and protozoa (4), the principal reason for the interest they have aroused has been the presence in these particles of ribonucleic acid. It is, in fact, this characteristic which has led to the idea that they might play a role in the synthesis of proteins and the differentiation of the embryo (4), has suggested possible analogies between these particles of the normal cell and the viruses, and has provoked the theory that they might be the plasmagens whose existence genetics is obliged to infer.

It should not, however, be overlooked that the cytoplasmic particles collected by ultracentrifugation of extracts of organs could have a chemical constitution different from that existing within the normal cytoplasm, resulting, for example, from the formation of complexes between these particles and the RNA at the instant of preparation of the extract. The error in interpretation we should be likely to commit if we did not take into account this possibility, did not escape the first experimentalists who isolated and studied the particles present in the extracts of tissues. Several have sought to avoid it by attempting to displace by centrifugation the cytoplasmic particles within the intact cells so that they would not thereafter be modified by any extraction technique. In particular, liver cells have been subjected to centrifugation for various lengths of time at various speeds by Brachet and Jeener (4), Claude (3), Chantrenne (22), and more recently by Brenner (23) and Lagerstedt (24). All have observed, in sections, the accumulation of RNA at the centrifugal pole of the cells. It would appear, therefore, that there exist, within the normal cell, some links between this substance and particles with speeds of sedimentation sufficiently high to be displaced. If the experiment is decisive in principle, it should nevertheless be pointed out that liver cells are crushed at the bottom of the tubes of the ultracentrifuge, are considerably deformed, and are placed under conditions as unfavorable as possible for their respiratory activity for periods varying from 10 min. to 2 hr. depending on the methods of the experimenter. It is somewhat difficult to believe that



the pH and the ionic concentration are not modified within the cytoplasm of cells thus treated.

Now, Kleczkowski (25) and Lauffer (26) have shown that a mixture of a nucleic acid (or of a nucleoprotein such as tobacco mosaic virus) with very different proteins gives rise to complexes separable by centrifugation, provided that the pII of the solution is intermediate between the isoelectric points of the two components. In addition, the bonds thus obtained may be broken simply by increasing the salt content of the solution, *i.e.*, precisely under the conditions where it is possible to separate a ribonucleoprotein of very small dimensions from various fractions of cytoplasmic particles obtained by the centrifugation of tissue extracts (4,27).

On the other hand, liver extracts contain an abundance of substances (fats, lipoprotein complexes?) which rapid centrifugation causes to accumulate at the surface of the liquid. The same phenomenon most likely takes place within the cell undergoing centrifugation. The region in which the RNA is localized following centrifugation could be a solution of RNA plus protein in water, the region deprived of RNA being occupied by substances of lower density. The experiments on the centrifugation of entire cells therefore furnishes a valuable, but not decisive, argument in favor of the presence of RNA in the cytoplasmic particles.

Doubts on this subject appear to have been removed by our study of the specific radioactivity acquired by the various fractions of cytoplasmic RNA during the experiments which are described above. In effect, if the RNA were to exist in the free state in the normal cell and were to attach itself to the cytoplasmic particles at the moment of preparation of the extract, the specific radioactivity of the RNA of all the fractions of cytoplasm should be the same. But, we have seen that, in the case of the liver cells, for example, this radioactivity varies from 1 to 7 from one fraction to another. It must therefore be admitted that, for reasons as yet unknown, the metabolism of RNA is influenced by its bonds with the cytoplasmic particles. These bonds therefore exist in the normal cell, where the presence of very small quantities of labeled orthophosphate cannot modify the physiology of the cell to any appreciable extent. It should be added that, if the RNA is considered as fixed to these particles, one is led to believe that these also exist within the cell.

If we now attempt to explain the differences observed between the specific radioactivities of the various fractions of RNA, nuclear as well as cytoplasmic, we leave the field of established conclusions for that of hypotheses and our efforts in this direction have no significance unless capable of experimental verification. We can, for the time being, limit ourselves to the following considerations:

1. Good arguments exist in favor of the idea that RNA is synthesized in the nucleus and then passes into the cytoplasm. In effect, when the liver cell or a nerve cell synthesizes RNA, this substance appears first in the nucleolus (24,28), then in the cytoplasm. In addition, it has been observed, on very different materials, that the cytoplasmic RNA appeared first in the immediate neighborhood of the nucleus (2,29,30,24), followed by its spreading through the entire cell.

2. When *Polytomella* cells, cultivated on a medium poor in phosphate (and containing, on this account, only small quantities of RNA), are placed in a medium the phosphate of which is normal, an appreciable quantity of RNA makes its appearance within a few hours in the immediate vicinity of the nucleus. This initial quantity of RNA comprises 95% of the fraction non-sedimentable in 60 min. at  $6 \times 10^4 \times g$ . Subsequently are formed the fractions linked to the particles, which soon become more important quantitatively than the free fraction (31). This striking fact is in accord with the hypothesis that ribonucleoproteins of very small dimensions are at the origin of the largest cytoplasmic particles, for which they serve in some way as a seed or which they build up by self-union (6, 27).

3. Finally, it should be noted that the slowing-down of protein synthesis in the cell is accompanied by a considerable decrease in the cytoplasmic content of RNA (32,33).

We are thus led to the theory that RNA is, for an important part at least, synthesized in the nucleus, passes into the region at the perimeter of the nucleus (where it is found in the form of molecules small enough to be non-sedimentable in 1 hr. at  $60,000 \times g$ ), spreads into the rest of the cytoplasm by integrating itself with cytoplasmic particles of large dimensions, and finally disappears.

If this is really so, one would expect that, shortly after the injection of labeled phosphate, the specific radioactivity of the RNA of liver cells would be stronger in the nucleus than in any of the cytoplasmic fractions, and stronger in the cytoplasmic fraction with lowest speed of sedimentation than in the other cytoplasmic fractions; also, that it would decrease in these latter as their speed of sedimentation goes up.

The distribution of the specific radioactivity between the various fractions of RNA of the liver is in accord with these ideas.

In the case of cells in active multiplication (embryos, *Polytomella*), any attempt at interpretation seems impossible in the present state of our knowledge.

## SUMMARY

Various fractions of RNA of rat liver, mice embryos, and *Polytomella* cells have been separated. The specific radioactivity acquired by the phosphate of the RNA of these fractions has been determined a short time after injection or introduction into the culture medium of phosphate labeled with  $P^{32}$ .

In nonmultiplying cells, this specific radioactivity is strongest in the nuclei. Within the cytoplasm, it decreases from the RNA fraction with the smallest speed of sedimentation to that with the highest speed of sedimentation.

In multiplying cells, the distribution of the specific radioactivity of the P of cytoplasmic RNA is the same, aside from the fact that the radioactivity of the RNA of the particles showing the highest speed of sedimentation is practically equal to that of the cytoplasmic RNA with the lowest speed of sedimentation.

An interpretation of these experimental facts is attempted for the case of nonmultiplying cells.

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# Effect of Repeated Doses of Gossypol on the Dog

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## INTRODUCTION

The reports that purified gossypol has an appetite inhibiting property (1) and has no generally toxic properties (2) have given rise to suggestions that this material might be used for clinical trial in the treatment of obesity. Gossypol is a polyphenolic, yellow pigment which is the principal component of the pigment glands of cottonseed (3). Its acute oral toxicity and that of the cottonseed pigment glands for rats, mice, rabbits, and guinea pigs have been reported (4). The present study describes the effect of repeated daily doses of highly purified gossypol at nine different dose levels on the food intake and body weight of 7 dogs.

## METHODS

In the first series of experiments 4 young, male dogs (litter mates weighing 5.9–6.4 kg.) were used. In the second series the surviving control dog from series one and 3 new adult female dogs (weighing 6.6–10.2 kg.) were studied. The dietary history and weight records of all 7 dogs were available since birth. The animals were maintained in an air-conditioned room on a daily diet of two parts of Swift's Dog Meal moistened with three parts water, on top of which was placed 50 g. fresh liver and 8 drops A and D oil. Water was allowed *ad lib*. Except for the 19 doses given by capsule during the first experimental period in series one, the remaining 186 doses were administered as an aqueous slurry by stomach tube prior to feeding. The food pans were inserted in a specially fitted block of wood so that loss of food from spillage was nil. All residual food was dried in an oven to constant weight and food consumption records were kept on a dry basis. The dry food values may be converted to wet food consumption by multiplying by the factor 2.6. The samples of highly purified gossypol used in this study were tested for their acute oral toxicity in the rat (5) and for their effect on the body weight of the rat after repeated daily doses by stomach tube at different dose levels (6).

## RESULTS AND COMMENT

The first series of experiments was conducted according to the following schedule: 55-day control period, 5-day experimental period (gossypol by capsule), 9-day control (rest) period, 5-day experimental period (gossypol by stomach tube), 9-day control period, 9-day experimental period (gossypol by stomach tube), and a final control period. A single dose of gossypol (84 mg./kg.) by capsule given in error to dog 1 (control) had no effect on the food consumption or body weight. In the experimental dogs, repeated doses of 50, 100, and 200 mg./kg. for the three experimental periods of 5, 5, and 9 days caused marked depression in body weight and food intake with immediate recovery of both after withdrawal of gossypol. Gossypol by capsule for 5 consecutive days at 50, 100, and 200 mg./kg./day proved only half as effective as when administered by stomach tube at the same dose levels for the same length of time. The maximal weight losses recorded for dogs 2, 3, and 4 occurred in the third period of gossypol administration (repeated daily doses of 50, 100, and 200 mg./kg., respectively, by stomach tube for 9 days) and were - 20, - 26, and - 25% of the body weight. The control dog lost 0.7% of his body weight during this same period. The average food intake (dry basis) of these experimental dogs fell from 289, 289, and 273 g./day for the preceding control period, respectively, to 46, 25, and 26 g./day for this experimental period; the control dog (no gossypol) had average daily food consumptions of 271 and 250 g./day for these two periods. The sudden death of all 3 experimental dogs in this series within 5 days after the last dose of gossypol prompted the warning note against the use of gossypol as an appetite depressant in human subjects (7).

The effects of smaller doses of gossypol were studied in series 2 over a period of 133 days. After a 45-day control period, dog 1 (5 mg./kg.), dog 5 (10 mg./kg.), dog 6 (1 and 15 mg./kg.), and dog 7 (2 and 30 mg./kg.) received 5, 12, 5, and 10 daily doses of gossypol by stomach tube with intervening control periods (no gossypol) of 9 days each. The smaller doses (1, 2, and 5 mg./kg./day) had only a slight effect on food intake and body weight. The larger doses caused much more striking effects. The greatest weight losses in dogs 5, 6, and 7 were in the fourth experimental period during which the animals lost 14, 11, and 17% of their body weight, respectively, after 10 successive daily doses of 10, 15, and 30 mg. gossypol/kg. body weight. Their respective

average daily food consumption values decreased from 239, 190, and 276 g./day for the preceding 9-day control period to 62, 63, and 44 g./day for the 10-day experimental period. During that same experimental period, dog 1, given only 5 mg./kg./day, showed a gain of 1.9% in body weight and a change from an average daily food intake of 244 g./day for the 9-day control period to 222 g./day for the 10-day experimental period. Despite apparently good recovery of food intake and body weight after cessation of gossypol intubation, dogs 5, 6, and 7 died, respectively, on the sixth, thirty-third and thirty-sixth day after the last dose of gossypol.

All 6 dogs which died had manifested lassitude, diarrhea, anorexia, and weight loss. Vomiting occurred only at the three highest dose levels (50, 100, and 200 mg./kg./day). Two of the dogs (Nos. 3 and 4) developed a conditioned reflex so that merely the sight of the stomach tube, syringe, observer, *etc.*, produced a very copious flow of saliva. Dog 1, the sole survivor, had received the least gossypol (5 mg./kg./day for four 5-day periods separated by 9-day rest intervals), did not develop diarrhea, maintained a constant average body weight and food consumption for eleven 10-day periods after the last dose of gossypol, and appeared normal in all respects.

Post-mortem examination of the 6 dogs showed consistent congestion of the splanchnic organs, marked gastroenteritis, excessive fluid in the abdomen (sometimes bloody), hydrothorax, edema of the lungs, and moderate to large amounts of fluid in the pericardium. The cecum, ileocecal valve, and portions of the intestine adjacent to the valve showed multiple round lesions (2-3 mm. in diameter) which appeared on the outside as greenish grey spots surrounded by a dark-red, circular, hemorrhagic zone. From the inside the solitary nodules were swollen and stood out prominently above the surrounding hyperemic mucosa, their centers punctuated by a small bleb of cloudy, mucoid material. The lesions were most numerous in the case of the solitary nodules in the lower part of the ileum. There was marked injection of the subserous blood vessels. In many cases the swollen nodules underwent partial necrosis and many of these areas were at the point of perforation.

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## SUMMARY

Repeated daily doses of gossypol by stomach tube at levels of 10, 15, 30, 50, 100, and 200 mg./kg. for periods varying from 5 to 12 days caused marked diarrhea, anorexia, and weight loss, and finally led to the death of 6 dogs. Repeated doses of 1, 2, and 5 mg./kg./day for similar periods produced only slight decreases in food intake and body weight and did not prove fatal.

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## Effect of Vitamin P Compounds on Choline Acetylase

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### INTRODUCTION

The mechanism by which certain flavonoid compounds, the so-called Vitamin P substances, act in the body is not clear. One method of attack on the problem has been the investigation of the effect of these substances on isolated enzyme systems. As a result of these investigations, Vitamin P compounds have been shown to exert inhibitory effects *in vitro* on the enzymes succinoxidase (1), hyaluronidase (2), and histidine decarboxylase (3,4). Bartlett (1) has postulated that the effect on succinoxidase is due to the quinone-forming properties of the flavonoids; this has been reported not to be the case with hyaluronidase (2) and histidine decarboxylase (4).

As part of a broad general program, a series of flavonoid compounds was tested to determine their effect on the enzyme, choline acetylase. This enzyme lends itself to such a study, since it has been reported to contain an -SH- grouping (5), and to be sensitive to quinones or quinone-forming compounds (9). In addition, the role of acetyl choline in spasticity and related pathologies suggested that a study on choline acetylase might prove to be of therapeutic interest.

### EXPERIMENTAL

Measurement of choline acetylase activity was carried out according to the method of Nachmansohn and John (5), as previously reported (6). Acetone-dried rat brain was used as a source of the enzyme. The dried powder was extracted with phosphate buffer (0.007 *M*, pH 7.4) containing KCl in 0.05 *M* concentration. Twenty ml. of this solution were used for the extraction of 1 g. of brain powder. Additions to the extract were choline chloride, final concentration 0.0025 *M*; sodium acetate, 0.02 *M*; sodium fluoride, 0.02 *M*; and cysteine hydrochloride, 0.015 *M*. The volume of these additions was about 0.25 that of the

total volume of extract plus additions. It was found that the addition of physostigmine produced little or no effect on the amount of acetylcholine formed, so that this was omitted.

The reaction was run in the Warburg apparatus. In the main compartment of the flasks were placed 2.5 ml. of the above solution and 0.5 ml. of a neutral solution of the substance being tested. The side bulb contained 0.3 ml. of a solution of adenosine triphosphate (ATP),  $3 \times 10^{-3} M$ . The Warburg flasks were filled with nitrogen and brought to temperature equilibrium at 37°. The ATP was then tipped into the main compartment, and the reaction was allowed to proceed for 20 min. At the end of this time the reaction was stopped by treatment with phosphate buffer, HCl, and NaOH (5), the precipitated protein was centrifuged off and the supernatant used for the assay.

The amount of acetylcholine formed was determined by measuring the contraction produced on the isolated guinea pig intestine by portions of the reaction mixture, and comparing it with the contraction produced by known amounts of acetylcholine. The tissue used was bathed in 50 ml. of Tyrode's solution, and was found to be sensitive to 0.1  $\mu g$ . of acetylcholine in this volume, with an optimum response at 1  $\mu g$ . of acetylcholine. Aliquots of the reaction mixture were therefore chosen so as to contain this amount. The total quantity of acetylcholine formed varied somewhat from one brain preparation to another, but averaged about 500  $\mu g$ . acetylcholine/g. of dried brain powder/hr.

With each experiment, controls were run to ascertain that the substance being tested had no effect on the testing tissue itself or on its response to acetylcholine. Results were computed by comparing the amount of acetylcholine produced in the reaction mixtures in the presence of flavonoid compounds with that produced in controls.

## RESULTS

The results are presented below in tabular form. The figures given are averages of the runs made. Acetylcholine formed is given in micrograms per gram of powder per hour.

The compounds tested were chosen so as to include as many as possible of the commonly occurring substances having Vitamin P activity, and whose solubility permitted testing under the conditions of the experiment. Quercetin and rutin, and esculetin and esculin provided examples of compounds of the same basic flavonoid structure in the glycosidic and aglycone forms.

## DISCUSSION

The results indicate that the inhibitory effect of the flavonoids examined is due to their quinone-forming properties. The structure of

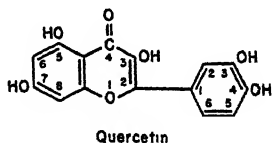
TABLE I  
*Effect of Vitamin P Compounds on Choline Acetylase*

Compound	Concentration	Acetylcholine formed		Inhibition
		Control	Experimental	
	<i>mg./ml</i>			<i>%</i>
Hesperidin methylchalcone	1.0	480	480	0
Carbethoxy hesperidin	1.0	530	530	0
Neohesperidin	1.0	515	510	0
Naringin	1.0	470	475	0
Rutin	1.0	500	300	40
Rutin	0.1	460	460	0
Quercetin	1.0	540	0	100
Quercetin	0.1	525	320	40
Esculin	1.0	500	505	0
Esculetin	1.0	495	0	0
Esculetin	0.1	495	255	50
4-Methylesculetin	1.0	500	200	60
4-Methylesculetin	0.1	450	440	0
D-Catechin	1.0	550	160	70
D-Catechin	0.1	550	550	0
D-Epicatechin	1.0	550	165	70
D-Epicatechin	0.1	480	180	0
Morin	1.0	430	0	100
Morin	0.1	520	180	65
3,4-Dihydroxyphenyl 3',4' methylene dioxystyryl ketone	1.0	475	0	100
3,4-Dihydroxyphenyl 3',4' methylene dioxystyryl ketone	0.1	480	180	0
2,4,6-Trihydroxyphenyl 3',4' methylene dioxystyryl ketone	1.0	530	450	15
2,4,6-Trihydroxyphenyl 3',4' dihydroxystyryl ketone	1.0	495	0	100
2,4,6-Trihydroxyphenyl 3',4' dihydroxystyryl ketone	0.1	600	605	0
p-Benzoquinone	1.0	500	100	80
p-Benzoquinone	0.1	520	520	0
Gentisic acid	1.0	485	290	10
Gentisic acid	0.1	490	490	0

the hesperidin derivatives tested and of naringin is such that they cannot form quinones; they were found to be inactive. Esculetin was highly active, but esculin, identical except for the blockage of one hydroxyl group, was completely inactive. The same change from

quercetin to rutin resulted in a loss in activity, although the rutin, with one potential quinone-forming site remaining, still showed some effect.

It would appear that, although two possible sites of quinone formation exist in many of these compounds, one grouping is appreciably more active than the other. The structure of quercetin is given below,



the active quinone-forming groups being the 3',4' dihydroxy and the 3-hydroxy 4-keto. In rutin, the 3-position is blocked, and the activity is about one-tenth that of quercetin. Catechin has no keto group at position 4, and the activity is again of the order of one-tenth that of quercetin. In morin, on the other hand, the dihydroxy grouping is 2',4', thus precluding quinone formation, and the activity is approximately the same as that of quercetin. The 3,4 grouping is thus apparently much more active than the 3',4'.

The three synthetic styryl ketone derivatives tested again illustrate the importance of quinone-forming groups. The one with no *o*-dihydroxy grouping was much less active than either of the others. In these compounds it seems to make no difference which of the rings contains the active grouping.

There would seem to be no relation between the action of the flavonoid compounds on choline acetylase and their Vitamin P properties. Esculin and esculetin have been reported to have the same Vitamin P activity (7), and show widely different effects on choline acetylase. Epicatechin, reported as an extremely potent Vitamin P substance, and catechin, without activity (8), have identical effects on the enzyme.

Whether the inhibitory effect demonstrated *in vitro* can be shown to occur in the body, and whether such an effect would be of therapeutic value are of course questions which only further investigation can answer.

## SUMMARY

Certain compounds of a flavonoid structure have been shown to inhibit the action of choline acetylase *in vitro*. This action is believed to be due to the quinone-forming properties of the compounds.

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# Heavy-Metal Hydroxides in *Statu Nascendi* as Reagents for the Purification of Amino Acid Mixtures and the Preparation of Pure Heavy-Metal Salts of Individual Amino Acids <sup>1</sup>

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## INTRODUCTION

The mercuric acetate-sodium carbonate reagent (1) is the only available reagent which will quantitatively separate amino acids from various contaminants commonly present in acid hydrolyzates of proteins. This reagent converts amino acids into mercury salts of carba-mino acids. These are precipitated by alcohol and from them the amino acids may be recovered by decomposition with  $H_2S$ . The method has been widely used both for the analysis of proteins and for the preparation of amino acids (2). For large scale work, however, the reagent is expensive and the bulky precipitate is inconvenient to handle.

The chief contaminants of the amino acids in acid hydrolyzates of proteins may be expected to include carbohydrates (3), humin, lipides, purines, etc. The presence of these products in amino acid mixtures prepared for physiological or therapeutic use is undesirable. In this paper we propose the use of nascent metallic hydroxides—particularly copper hydroxide—for the removal of non-amino acid material.

The important point in the proposed procedure is the formation of the metallic hydroxide within the solution by addition of copper sulfate followed by the calculated equivalent of barium hydroxide. To avoid excess of metallic hydroxide, a preliminary test of the amount needed to form complexes with the amino acids is made and a small excess

<sup>1</sup> Based in part on U. S. patent applications Serial Nos. 529,550 (April 4, 1944) and 642,431 (January 19, 1946), and U. S. Patent 2,462,124 (February 22, 1949).

<sup>2</sup> Deceased December 1947.

only is added in the main experiment. Nascent nickel and cobalt hydroxides act like nascent copper hydroxide, and may be used; but they are more expensive and consequently are not so desirable. Similarly, zinc, manganese, and cadmium may also be used; but they frequently give sticky salts, which are difficult to handle, and so are less useful technically.

The metallic derivatives of the amino acids may then be crystallized directly or after preliminary precipitation with an organic solvent, preferably acetone. The free amino acids are recovered by decomposition of the complexes with  $H_2S$ . Unlike amino acid mixtures purified by the usual methods, these preparations reflect rather closely the amino acid composition of the proteins from which they were derived and are suitable for nutritional studies. Of course, the tryptophan and a part of the hydroxy and thio amino acids have been destroyed during prior acid hydrolysis.

The small excess of copper hydroxide removes humin, other colloidal impurities and some non- $\alpha$ -amino acid material. Although the copper complexes of certain of the amino acids are insoluble in water they are effectively held in solution by the copper compounds of other amino acids.

The details and principle of the method are illustrated by the following typical experiments.

## EXPERIMENTAL

### *Part I*

1. One hundred g. casein is added to 600 ml. of 35% sulfuric acid, and kept at water-bath temperature, until the main part is dissolved. Then the mixture is boiled under reflux for 16 hr. An alternative procedure is to heat for 6 hr. in the autoclave at  $110^\circ C$ .

The hydrolyzate is neutralized with the calculated quantity of barium hydroxide solution; both solutions should be hot for better precipitation of  $BaSO_4$ . For obtaining good yields, it is essential to boil out the  $BaSO_4$  precipitate at least once more with about 500 ml.  $H_2O$ . After filtering, the solution which was dark purple or brown before, is light-colored, the  $BaSO_4$  precipitate having adsorbed large quantities of dark-colored by-products.

If significant amounts of  $NH_3$  are present they are removed at this point by the usual treatment with  $Ba(OH)_2$  or  $BaCO_3$ . This is not necessary when the  $NH_3$  content is small. In the course of the process, probably during the later treatment with acetone or alcohol-ether, the ammoniacal copper hydroxide\* formed is decomposed

\* Incidentally an ammoniacal cupric hydroxide solution (*Schweizer's reagent*) free from foreign ions may be prepared in the simplest way by an analogous procedure from barium hydroxide and  $(NH_4)_2SO_4$  or amine sulfates such as, *e.g.*, ethylene-

in such a way that the greater part of the ammonia remains with the organic solvents used for the precipitation reaction.

The capacity of this solution (which may contain traces of barium) to dissolve  $\text{Cu}(\text{OH})_2$  is determined in the following way: 10 ml. are diluted to 400 ml., an excess of basic cupric carbonate is added, and the mixture kept on the water-bath for several hours. Then, it is boiled for some time, filtered, the excess  $\text{CuCO}_3 \cdot \text{CuO}$  washed carefully, filtrate and washings brought to 250 ml., and  $\text{Cu}^{++}$  determined iodometrically. Now,  $\text{Cu}(\text{OH})_2$  is formed in the main portion, as follows: A quantity of  $\text{CuSO}_4$ , amounting to 110% of the capacity for Cu-uptake, calculated from the analysis, is dissolved in the amino acid mixture, in the cold or under gentle heating, and an equivalent amount (to  $\text{CuSO}_4$ ) of  $\text{Ba}(\text{OH})_2$  solution is added. Immediately, the deep blue solution of the Cu-compounds of amino acids is formed. The mixture is allowed to stand for about 48 hr.; in addition to  $\text{BaSO}_4$  and the excess of  $\text{Cu}(\text{OH})_2$ , there are Cu-salts of by-products and other impurities carried down in the precipitate. At the same time, reducing substances which may be present, are decomposed, forming a small quantity of  $\text{Cu}_2\text{O}$ . Phosphoric acid produced during hydrolysis when nucleic acid impurities are present is removed as the alkaline earth or copper salt, and purines may be precipitated by  $\text{Cu}_2\text{O}$ . After filtering and reducing *in vacuo* to about 250 ml., the solution is poured into 4500 ml. acetone. Immediately, the main part of the amino acids (83–85%) is precipitated in the form of their light-blue Cu-compounds. The powdery precipitate may be filtered off by suction, or centrifuged at once. It is washed with acetone, and forms fraction I.

The acetone mother liquor is concentrated *in vacuo* to 50 ml., acetone being recovered. To this concentrate, from which a part of the remaining Cu-salts starts crystallizing, an equal volume of ethanol, containing 10–15% ether, is added with vigorous shaking. The Cu-salts which have been dissolved in acetone, are precipitated so completely now, that the filtered solution is only slightly blue, and is discarded. The precipitate, washed with ethanol–ether mixture, forms fraction II.

The combined fractions I and II are dissolved in 1 l. water, and the Cu-salts are decomposed with  $\text{H}_2\text{S}$ . The filtrate from  $\text{CuS}$  is colorless or only slightly yellow; it is allowed to stand in the refrigerator for 24 hr.; most of the tyrosine separates as crystals of a surprising purity, and is filtered off. The solution is now freed from last traces of Ba with  $\text{H}_2\text{SO}_4$ . After complete evaporation *in vacuo*, the amino acids remain as a very slightly yellowish mass, and can be ground to a white powder, clearly soluble in water to 8–10%. The solution has a pH of about 5, and is stable.

Yield of dry amino acids: 85–88 g. Total N: 14.5%. Even a concentrated solution gives no biuret reaction.

Any water-soluble ketone, ether, and some alcohols as well as cellosolve may be used as precipitant for the copper salts of the amino acids; for maximum separation, ketones as, e.g. acetone and methyl ethyl ketone, and the cyclic ether dioxane are very desirable. However, in many cases, we found that the step of solvent precipitation

diamine sulfate. *Eau celeste*, the deep blue solution resulting from  $\text{CuSO}_4 + \text{NH}_4\text{OH}$  is described by H. Baubigny [*Compt. rend.* 104, 1616 (1887)] as differing from Schweizer's reagent in its properties. Our procedure is preferable to the frequently used time-consuming method of R. A. Toyner [*J. Chem. Soc.* 121, 1512 (1922)]. This author recommends treatment of copper turnings with ammonia in an air stream in the presence of saccharose catalyst.



may be eliminated. The behavior of the Cu-salts, as described above, was unexpected. Some Cu-salts of amino acids are known to be only very slightly soluble in water, for example, the Cu-salts of cystine, aspartic acid, or tyrosine. In the mixture, however, they are kept in solution by action of the highly soluble copper derivatives of the diamino acids, and of certain aliphatic  $\alpha$ -amino-monocarboxylic and hydroxy amino acids. This remarkable effect may also be observed on an artificial mixture of amino acids.

2. In place of the  $\text{Ba}(\text{OH})_2$  of Example 1, freshly prepared  $\text{Ca}(\text{OH})_2$  is used for neutralization of the sulfuric acid, and nascent  $\text{Cu}(\text{OH})_2$  is prepared in a similar manner with lime instead of barium hydroxide. Having determined the necessary amounts of copper ion, molar equivalents of  $\text{CuSO}_4$  and  $\text{Ca}(\text{OH})_2$  are added and the hydrolyzate is left standing, for about 24 hr., at room temperature or under refrigeration. After removal of the precipitated solids, the amino acid-copper salts contained in the liquid are decomposed with  $\text{H}_2\text{S}$ . The resulting cupric sulfide, which apparently is able also to carry down impurities that may be left still in the hydrolyzate, leaves, after being removed, an amino acid solution that is substantially pure, save for small quantities of  $\text{CaSO}_4$  which stay in solution but may easily be eliminated with  $\text{Ba}(\text{OH})_2$  and oxalic acid. The hydrolyzate may now be utilized either as such or be used for the preparation of crystalline amino acids.

3. One hundred g. blood protein are hydrolyzed, by refluxing, for about 16 hr. with hydrochloric acid having a strength of 20% by weight of the hydrolyzate mix. The extract, after being separated from the solids remaining after, or having formed during, the hydrolysis, is concentrated *in vacuo* to preferably sirupy consistency while simultaneously being freed of most of the hydrochloric acid. For the removal of hydrochloric acid not eliminated during the concentration *in vacuo*,  $\text{PbCO}_3$  or  $\text{Cu}_2\text{O}$  may be used. We find it convenient, however, to utilize ion-exchange materials (such as Deacidite or Amberlite IR-4) which permit the removal of mineral acid without any loss of essential amino acids. The procedure of purifying and separating amino acids from foreign substances is then carried out in the same manner as illustrated in Examples 1 or 2. An end product, substantially enriched in indispensable amino acids and thus of superior utility for certain purposes, may be obtained by regulating the above ion-exchange process in such a manner that glutamic and aspartic acids, two nonessential constituents, are eliminated from the amino acid mixture.

4. Same procedure as in Example 1; the purification is carried out using the nickel salts instead of the copper salts. Ni-uptake is determined in analogous manner by boiling with basic nickel carbonate, and determination of dissolved Ni with dimethylglyoxime. Formation of  $\text{Ni}(\text{OH})_2$  from  $\text{NiSO}_4$  and  $\text{Ba}(\text{OH})_2$  is analogous, and from this step on the procedure is identical with that described in Examples 1 or 2.

In the same way, cobalt may be used. Cobalt and nickel form solid amino acid salts, whereas zinc, manganese, and cadmium hydroxides yield sticky metal salts of the amino acid mixture. None of these metals is as advantageous as Cu.

Any other protein may be used in place of casein or blood protein.<sup>4</sup> Of course, the

<sup>4</sup> The suitability of the hydrolyzates prepared and purified as described and possibly fortified with tryptophan for both oral and parenteral application has been confirmed by clinical tests carried out at the Biochemical Division of Interchemical Corp., Union, N. J.

final amino acid mixture does not contain tryptophan, which is destroyed by the acid hydrolysis. The process, however, can be applied to any enzymatic hydrolyzate yielding products giving a negative biuret test. In spite of its low solubility *L*-tryptophan-copper (see below) then present dissolves in the copper salt mixture. Lower peptides have no harmful effect. According to Abderhalden and Fodor (4) they too combine with copper hydroxide.

### Part II

Another reaction based on the action of metallic hydroxides *in statu nascendi* is that recommended many years ago by Neuberg and Mayer (5) for the preparation of difficultly soluble amino acid salts which could not be readily obtained by other means. This method involves the precipitation of a solution of an amino acid in the exactly equivalent amount of alkali hydroxide with a soluble heavy-metal salt. The corresponding heavy-metal salt of the amino acid is obtained and not the metal hydroxide. Thus the authors have been able to prepare the normal cupric and mercuric salts of cystine by precipitating the sodium salt with cupric sulfate and mercuric acetate. We have found that most of the difficultly soluble salts of amino acids can be directly precipitated in an analogous manner. The preparation of the nickel salt of isoleucine and of the copper salt of tryptophan are described below as typical examples. The latter salt can otherwise only be obtained by complicated procedures.

The precipitation of proteins in solution or suspension by means of cupric hydroxide (6) follows another mechanism. In this case, the action of the reagent is comparable to that of colloidal ferric or aluminum hydroxide.

### Tryptophan-Copper

When an aqueous solution of tryptophan is boiled with freshly precipitated and finely dispersed cupric hydroxide all the tryptophan settles down as a copper precipitate together with the excess reagent, and a colorless supernatant liquid results. Careful treatment with dilute HCl leads to removal of the excess  $\text{Cu}(\text{OH})_2$ . In this way Abderhalden and Kempe (7) obtained the copper salt of *L*-tryptophan in the form of a greyish-blue amorphous powder.

A simpler procedure to obtain the same compound directly, instantaneously, and in crystalline form has now been found:

Two g. *L*-tryptophan is dissolved by shaking in 10 ml. 1 *N*  $\text{NaOH}$ . The desired derivative is then precipitated from the clear solution—previously filtered or centrifuged if necessary—by means of 26 ml. 0.2 *M*  $\text{CuSO}_4$  or  $\text{CuCl}_2$ . Filtering off by suction, washing with water till free from anions, and drying under vacuum gives blue to violet crystals (small needles) in quantitative yield.

Cu calculated for  $(\text{C}_{11}\text{H}_{11}\text{N}_2\text{O}_2)_2\text{Cu}$ : 13.5%; Cu found: 13.6%.

*DL*-Tryptophan-Copper is prepared in an exactly analogous manner and resembles the derivative of the *L*-form in all particulars.

*Isoleucine-Nickel*

In an analogous manner the nickel salt of DL-isoleucine was prepared from 1.5 g. of the amino acid in 10 ml. 1 *N* NaOH and 26 ml. 0.2 *M* NiSO<sub>4</sub> or NiCl<sub>2</sub>. Filtering after several hours standing, a quantitative yield of pale-blue crystalline leaflets was obtained.

Ni calculated for (C<sub>6</sub>H<sub>11</sub>O<sub>2</sub>N)<sub>2</sub>Ni: 18.4%; Ni found after drying to constant weight: 18.3%.

The racemate is soluble in methanol just like L-isoleucine-nickel (8).

## COMMENTS AND SUMMARY

Heavy-metal salts of many amino acids, in particular copper salts, are difficultly soluble and have therefore been used for the identification and separation of individual amino acids (leucine, aspartic acid, glutamic acid, cystine, phenylalanine, tyrosine). These practically insoluble derivatives become soluble when formed in the presence of other amino acids. The difficultly soluble salts are held in solution<sup>5</sup> by the readily soluble copper salts of the lower aliphatic amino acids, hydroxy amino acids, proline and basic amino acids. What is true for the copper compounds equally applies to other salts, *e.g.*, the nickel derivatives.

Formerly copper salts of amino acids were made by boiling a usually highly dilute solution with copper hydroxide or copper carbonate or occasionally, for analytical purposes, copper phosphate (11). Preparation of copper hydroxide or basic copper carbonate free from harmful admixtures is a difficult and tedious task; combination of all the amino acids with copper requires hours of boiling (12) and repeated evaporation with a large excess of basic CuCO<sub>3</sub>. The copper sludge must be extracted with much water, then filtered off and fresh copper reagent added to the filtrate which is again evaporated to a sirup. This treatment must be repeated three or more times preferably with constant mechanical stirring of the copper carbonate suspension. The necessity of this complicated procedure is clearly stated by Town, Brazier, Damodaran, and by Stockelbach and Bailey (12). Furthermore the process can only be successfully accomplished with freshly prepared copper reagents. Inconveniently, these are gelatinous or voluminous.

<sup>5</sup> This may be due to formation of cupric double salts like those observed by Fischer (9) in the case of valine and leucine and by Town (10) for glutamic and aspartic acids. The copper derivatives of the two related amino acids give mixed crystals. [See also (11).]

The simple procedure described circumvents all these disadvantages. It is based on the instantaneous formation of cupric hydroxide, in reactive form and free from all impurities, directly in the amino acid solution. *In statu nascendi*, the cupric hydroxide *immediately and without heating* combines with all the amino acids to complete saturation. (When  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  is dissolved in a mixture of amino acids the deepening of the blue coloration indicates complex-formation. The  $\text{SO}_4^{--}$  reaction is not affected, however.) Addition of a saturated  $\text{Ba}(\text{OH})_2$  solution precipitates all the  $\text{SO}_4^{--}$  ions as  $\text{BaSO}_4$ ; at the same time the supernatant liquid turns a deep blue—the color of complex cupric amino acid solutions. In the course of its formation the  $\text{BaSO}_4$  carries down as a co-precipitate the admixed impurities present in yellowish-brown protein hydrolyzates.

This useful effect causes water-clear solutions of the amino acids to be regenerated from their copper derivatives. (The  $\text{CuS}$  formed during decomposition of the copper salt with  $\text{H}_2\text{S}$  has the same effect. Both precipitates can be washed without difficulty so that no significant loss of amino acids is sustained.)  $\text{Ba}(\text{OH})_2$  may be replaced by  $\text{Sr}(\text{OH})_2$  or  $\text{Ca}(\text{OH})_2$ . The only important point is the availability of the cupric hydroxide *in statu nascendi*. This condition is equally complied with in the method described for the preparation of pure heavy-metal salts of individual amino acids.

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# Spectrophotometric Studies of the Oxidation of Fats.

## IX. Coupled Oxidation of Vitamin A Acetate <sup>1</sup>

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### INTRODUCTION

The vitamin A activity of oils and concentrates decreases with age and upon exposure to air. This loss has been recognized as an oxidative process which is hastened by irradiation, an oxidation coupled with that of the polyunsaturated fatty acids present in the mixture. The changes occurring in the light-absorption characteristics of such mixtures as a result of the oxidation processes have received considerable study (1-4). The kinetics of the coupled oxidation of carotenoid pigments related to vitamin A in a medium of oxidizing linoleate esters have received some attention in the hope that such studies would throw some light on the mechanism of the oxidation. The coupled oxidation of carotene (5) and of bixin (6) have been studied and the spectral changes have been related to the oxygen uptake of the systems. These substances suffer the disadvantage that they are only slightly soluble in common solvents suitable for spectrophotometric work, therefore studies of the autoxidation of these substances themselves is prohibited under conditions comparable to those in the coupled oxidation. Vitamin A acetate is not subject to this disadvantage, and it lends itself to a study of its autoxidation as well as to its coupled oxidation in methyl linoleate. Therefore it was chosen for a study of the chromophore changes accompanying the coupled oxidation in linoleate and the autoxidation of the vitamin itself.

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## EXPERIMENTAL

The experimental conditions used in this study have been identical to those used in the preceding papers of this series (5, 7), with the aim that the results of the coupled oxidation be comparable to those of autoxidation of the linoleate carrier. Oxidations were carried out in Warburg vessels at 37° under air. For the coupled oxidations a mixture of 0.775% vitamin A acetate (generously donated by Distillation Products, Inc.) in highly purified methyl linoleate (Hormel Foundation) was prepared. Samples taken for the oxidation were approximately 250 mg. in size. The samples were removed from the Warburg apparatus after convenient intervals of oxygen absorption and were dissolved and diluted with ethanol immediately before the ultraviolet absorption was determined. The autoxidation of vitamin A acetate was studied using samples containing 63.8  $\mu$ moles of vitamin A acetate dissolved in 1.0 ml. redistilled triacetin per Warburg vessel. The procedure used in these experiments was identical to that described for the coupled oxidation series.

## RESULTS AND DISCUSSION

The spectra of the samples taken during the course of the coupled oxidation (Fig. 1) are very similar to those shown by other investigators (1-4). The decrease in light absorption by the principal chromophore of the vitamin A acetate is accompanied by an increase in the absorption in the region of 2350 Å due to the oxidation products of the linoleate (7). When the light absorption at 3280 Å (vitamin A acetate) and at 2325 Å (products of linoleate oxidation) are plotted against the oxygen uptake of the system in Fig. 2, it is seen that in the early stage of the oxidation the diene chromophore produced from linoleate by oxidation bears a linear relationship to oxygen content, analogous to the results reported for linoleate itself (7), carotene in linoleate (5), and bixin in linoleate (6). The destruction of vitamin A acetate also follows a course similar to that of the destruction of carotene and bixin. When the logarithm of  $E_{1\text{cm}}^{1\%}$  at 3280 Å is plotted against oxygen uptake, the points lie on a straight line indicating the destruction of vitamin A acetate, like carotene and bixin, is a logarithmic function of the oxygen uptake of the system. As in the cases of these carotenoids, when 10% of the linoleate has been oxidized, nearly all of the vitamin A has been oxidized. This emphasizes the importance of protection of vitamin-A-active oils against even traces of oxidative rancidity.

In Fig. 3 the course of the autoxidation of vitamin A acetate alone is plotted against time. The oxygen absorption follows the typical sigmoid curve of autocatalytic reactions, and the primary reaction appears to

involve only 1 mole of oxygen per mole of vitamin A acetate, for the reaction slows down considerably when this point is reached. The extinction coefficients at 3280 Å and 2425 Å are related to oxygen uptake of the system in Fig. 4. The extinction coefficient at 2425 Å, a minor chromophore in vitamin A acetate, increases slightly and in proportion to the oxygen uptake of the ester. However, the increase

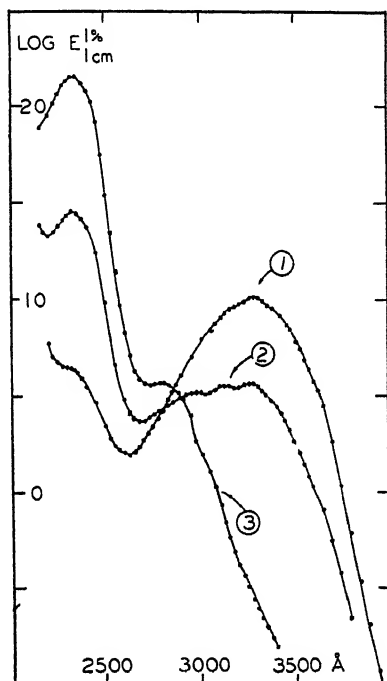


Fig. 1. Spectra of vitamin A acetate in methyl linoleate during the course of the autoxidation. 1. Fresh sample; 2. 0.0481 moles oxygen per mole ester; 3. 0.197 moles oxygen per mole ester.

is so slight that the contribution of oxidized vitamin A acetate to the increase in absorption in the conjugated diene range in coupled oxidations is negligible. The extinction coefficient at 3280 Å, on the other hand, decreases sharply in a logarithmic relationship to the oxygen uptake. This logarithmic relationship exists as far as 1 mole of oxygen per mole ester.



It seems likely that the products of oxidation of vitamin A acetate are similar in the two types of oxidation. If this be true, the increase in absorption at the conjugated diene region in the coupled oxidation system contributed by the oxidized vitamin A acetate is inconsequential, in the order of 0.2% of the total. Making correction for this contribution, the apparent molar extinction coefficient for the primary product of oxidation of the methyl linoleate was calculated to be 22,220. Bolland and Koch (8) found a value of 22,700 (70% conjugated).

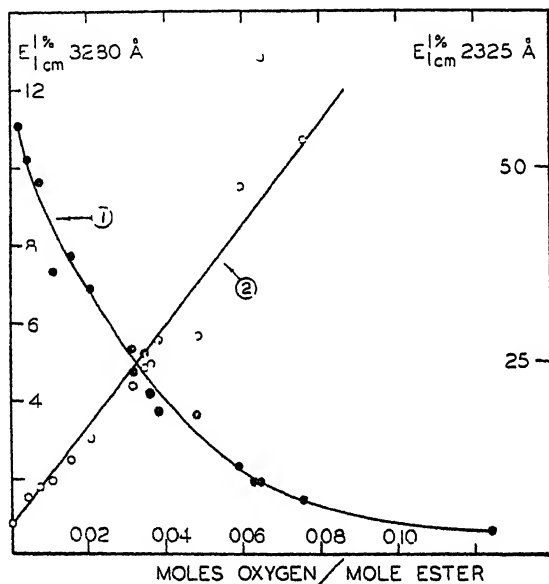


FIG 2. The course of the coupled oxidation of vitamin A acetate in methyl linoleate. 1. Vitamin A acetate destruction; 2. Diene conjugation.

tion) for the autoxidation of ethyl linoleate at 45°, and Lundberg and Chipault (9), on the basis of critical experiments on very highly purified linoleate concluded that the peroxide of linoleate was 70% conjugated, irrespective of the temperature at which the oxidation took place. It thus appears that the main course of the oxidation of linoleate is unaffected by the presence of this amount of vitamin A acetate, for the proportion of conjugated to nonconjugated peroxides is the same whether the vitamin A acetate be present or not. This is

likewise true for systems containing carotene (5) in which an extinction coefficient of 22,700 was observed.

It appears that the mechanism of oxidation of vitamin A acetate is similar to that of bixin (6) and carotene (5) in coupled oxidation with linoleate. The vitamin A acetate might first be attacked by either (1).

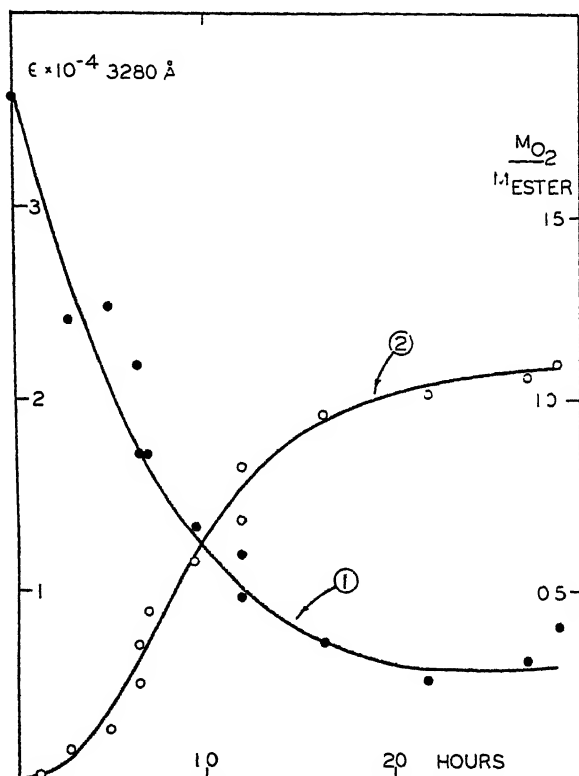


FIG. 3. The course of autooxidation of vitamin A acetate (no linoleate present). 1. Destruction of principal chromophore ( $\epsilon$  at 3280 Å). 2. Oxygen consumption, moles oxygen per mole ester.

supplying a hydrogen to a linoleate peroxide free radical or, (2) by the addition of free radicals to the polyene system. The possible primary products of the oxidation of vitamin A acetate having fewer than five conjugated double bonds do not appear to accumulate. There is evidence for a small amount of such compounds in the spectra of

partially oxidized samples such as is shown in Fig. 1, curve 2. However, the sample in which the vitamin A is virtually completely oxidized (curve 3) has a spectrum very similar to that of ethyl linoleate alone at approximately the same degree of oxidation (7).

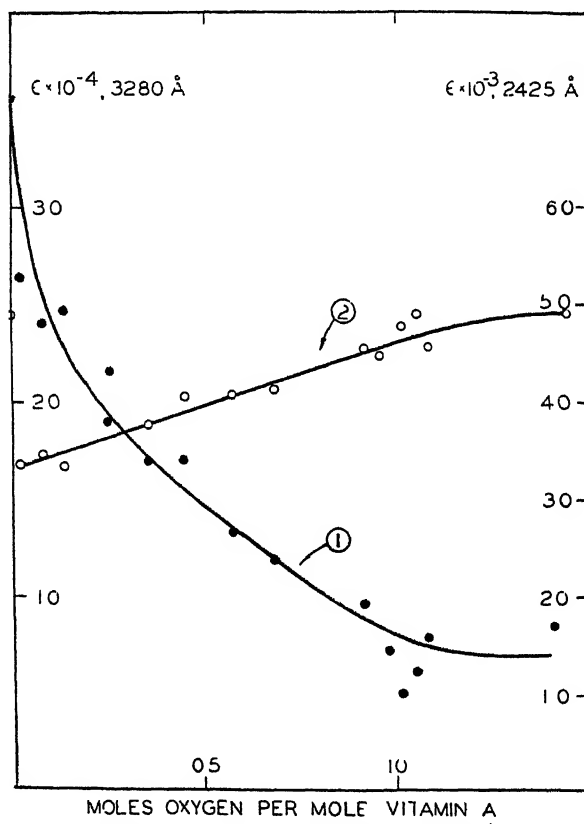


FIG. 4. Chromophore changes in vitamin A acetate autoxidation as a function of oxygen consumption (no linoleate present). 1. Principal chromophore,  $\epsilon$  at 3280 Å. 2. Light absorption in conjugated diene range,  $\epsilon$  at 2425 Å.

The change of the principal chromophore of vitamin A acetate with oxidation is similar to that of the decrease in extinction at 2680 Å in autoxidizing  $\beta$ -eleostearic acid as studied by Brauer and Steadman (10). This conjugated polyene shows a logarithmic decrease in its

principal chromophore consequent to oxidation as do the carotenoid pigments. These authors concluded that in the oxidation of the conjugated triene a conjugated diene is formed which in turn is oxidized. Similar reactions may take place in the vitamin A acetate oxidation, but far more detailed study must be made before a mechanism of oxidation of vitamin A acetate can be postulated.

### SUMMARY

The coupled oxidation of crystalline vitamin A acetate in highly purified methyl linoleate has been studied from the point of view of oxygen uptake and spectral changes. A parallel study of the autooxidation of vitamin A acetate alone has also been made. It appears that the products of oxidation of vitamin A acetate are spectrally similar in the two oxidations. As in the cases of carotene and bixin, vitamin A acetate is virtually destroyed before 10% of the linoleate carrier is oxidized, emphasizing the importance of protection of vitamin A bearing oils from even traces of oxidation. The course of oxidation of linoleate is unaffected by the presence of vitamin A acetate.

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# The Enzymatic Hydrolysis of L- and D-Amino Acid Amides by Mushroom Preparations

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## INTRODUCTION

L-Leucine amide and L-alanine amide are rapidly hydrolyzed by preparations of animal tissues from several sources to yield the free amino acid and ammonia (1-7). Under the same conditions, the D-forms of these amides are not at all attacked. Many other amino acid amides, such as glycine amide (1,5,7),  $\alpha$ -aminobutyric acid amide (5,7), valine amide (5), norleucine amide (5,7), histidine amide (8), S-benzylcysteine amide (8), tryptophan amide (7), isoglutamine (3,9), isoasparagine (10), and  $\alpha$ -benzoylarginine amide (11), are also readily hydrolyzed by various kinds of animal enzyme preparations. In contrast, the amides of proline and of hydroxyproline are almost completely resistant to the action of any of the animal enzyme preparations studied (2,8).

We have confirmed the fact that several animal tissues effect little or no hydrolysis of proline amide. We have noted, however, that proline amide is readily hydrolyzed by crude aqueous extracts of mushrooms.<sup>1</sup> Of still further interest is the fact that such extracts hydrolyze both optical isomers of DL-proline amide. The properties of the mushroom extract are therefore doubly unusual, for in contrast with those animal tissue extracts studied, the mushroom extract not only readily hydrolyzes L-proline amide but also the D-isomer thereof. We have further noted that mushroom preparations likewise hydrolyze both optical isomers of DL-alanine amide and of DL-leucine amide. To our knowledge, these are the first observations on the enzymatic hydrolysis of D-amino acid amides.

<sup>1</sup> Proline amide is also slightly hydrolyzed by fresh brewers' yeast.

## EXPERIMENTAL

*DL-Proline Amide*

One hundred and fifteen grams of L-proline was dissolved in 1 l. of boiling glacial acetic acid, 224 g. of acetic anhydride (2.2 moles) was added, and the solution allowed to stand at 25° for 2 hr. On evaporation *in vacuo*, a yellow sirup was obtained which was brought to crystallization by rubbing with acetone. The product, which was nearly pure acetyl-DL-proline, was recrystallized from acetone-ether. M. p. 106°, N: calculated, 8.9%; found, 8.8% (12). The yield of pure acetyl-DL-proline was 98 g., or 63% of the theory. A 4% solution of the compound in water gave no measurable optical rotation. The acetyl group was removed by refluxing the compound with the 10-fold volume of 2 N hydrochloric acid for 2 hr. Removal of the solvent *in vacuo* gave a nearly quantitative yield of DL-proline hydrochloride.

One hundred and five grams of DL-proline hydrochloride were dissolved in 1 l. of absolute alcohol, and the solution saturated with hydrochloric acid gas. The esterification mixture was evaporated *in vacuo* to a thick sirup, the residue dissolved in ice water, and the chilled aqueous solution layered with ether. A 5 N sodium hydroxide solution was added dropwise with shaking until the mixture was alkaline to litmus. Solid, anhydrous potassium carbonate was then added until a thick sludge was formed. The latter was extracted several times with ether, and the combined ethereal extracts were dried over anhydrous sodium sulfate. After removal of the ether, the racemic proline ester was purified by fractional distillation *in vacuo*. Seventy-four grams of ester was collected at 50–70° at 5 mm. Hg pressure. The ester was dissolved in methanol saturated at 0° with ammonia. After 6 days at 25°, the solvent was removed *in vacuo*, and the residue, which was nearly entirely DL-proline amide, was recrystallized from chloroform in the form of glistening plates. M. p. 99°. N: calculated, 24.5%; found, 24.3%. Yield: 36 g. L-Proline amide hydrochloride has been prepared by Smith and Bergmann by the carbobenzoxy procedure (2).

*Action of Animal Tissue Preparations on DL-Proline*

Rat liver, kidney, or intestinal mucosa homogenates, incubated at pH 8.0 with DL-proline amide, produce little or no hydrolysis of this substrate. The same can be said of homogenates of hog pancreas. The maximum rate of hydrolysis of the substrate at 38° by these tissues is invariably less than 1  $\mu$ mole/hr./mg. N.<sup>2</sup> Both liver and kidney homogenates readily hydrolyze the amides of leucine and of alanine (5).

*Mushroom Preparations and Their Action on DL-Proline Amide*

The mushrooms employed were the white, edible variety (*Psalliota campestris*), grown by C. L. Powell & Son, Kennett Square, Pennsylvania, and purchased from a local food market.

<sup>2</sup> We have noted, however, that slices of rat kidney or of rat liver appreciably metabolize DL-proline amide. Somewhat more than 2 moles of ammonia are evolved per mole of racemic amide. Whether the L-isomer is desamidated and then oxidized [cf. (13,14)], or whether both isomers are desamidated remains for further investigation.

Active fractions of this material were prepared as follows. Fresh mushrooms (4740 g.) were ground in a Waring Blendor with 14,220 ml. of ice-cold water and allowed to extract at  $+ 5^{\circ}$  overnight. The next morning it was put through gauze and then filtered by gravity on Schleicher & Schüll No. 588 12.5-cm. paper. The volume of  $S_1$  was 15,900 ml. and its pH 6.6. Its activity was 16  $\mu$ moles/hr./mg. N.<sup>3</sup> The temperature was lowered to  $0^{\circ}$ , and 80% alcohol was added to a final concentration of 30%, during which time the temperature was lowered progressively to  $-15^{\circ}$ . By the following morning a precipitate had settled to the bottom. The supernate was siphoned off and the precipitate collected at  $-15^{\circ}$  in a Sharples centrifuge at  $62,000 \times g$  (to the outer wall of the centrifuge bowl). The precipitate,  $P_2$ , was suspended in water; it had a total N of 1470 mg. and an activity of 27  $\mu$ moles/hr./mg. N. The supernatant,  $S_2$ , was maintained at  $-15^{\circ}$  and its pH lowered to 4.8.<sup>4</sup> After 24 hr. a precipitate had formed and settled to the bottom. The supernate was siphoned off and the precipitate collected at  $-15^{\circ}$  in the Sharples. This step requires careful control of temperature. The brownish black precipitate,  $P_3$ , is small in amount but is very active, with a total N of 390 mg. and an activity of 154  $\mu$ moles/hr./mg. N.<sup>5</sup>

### *pH-Activity Relation*

The relation between pH and susceptibility of proline amide to hydrolysis by the purified mushroom fraction  $P_3$  is shown in Fig. 1. The maximum susceptibility occurs at about pH 8.0. This presumably relates to the hydrolysis of the L-form which is measured under these conditions.

### *Maximum Hydrolysis of the Amides*

Various solutions of DL-proline amide, DL-alanine amide (1,8), and DL-leucine amide were incubated with purified preparations of mushrooms and the incubation continued until both optical forms of each amide were hydrolyzed. At intervals during this period, portions of the digests were removed and analyzed both for ammonia N and for carboxyl N by the ninhydrin- $\text{CO}_2$  procedure. The data are given in Fig. 2.

Up to 50% hydrolysis of the racemates, the enzymatic cleavage is rapid. Beyond the 50% point, the rate is some 30 times slower. The fast rate refers presumably to that of the L-form of the amides, the slow rate to that of the D-form.

<sup>3</sup> The rate of activity of the various fractions is expressed in terms of  $\mu$ moles of an 0.05 M solution of DL-proline amide hydrolyzed/hr./mg. N at  $38^{\circ}$  and a pH of 8.0. These rates were approximated from the initial portion of the respective time curves and refer to the rate at which the most rapidly hydrolyzed isomer, presumably the L-form, was cleaved. The activity of these preparations against the more slowly hydrolyzed isomer can only be roughly estimated with the racemic substrate.

<sup>4</sup> All pH determinations were taken on aliquots diluted to a final concentration of 5% alcohol and were measured with a glass electrode.

<sup>5</sup> Other amino acid amides hydrolyzed by crude, aqueous mushroom extracts are glycine amide, L-isoglutamine, and L-isoasparagine. The rate of hydrolysis of these substrates is however considerably less than those of the amides of proline, alanine, and leucine. L-Glutamine and L-asparagine appear to be completely resistant when incubated with preparations of mushrooms.

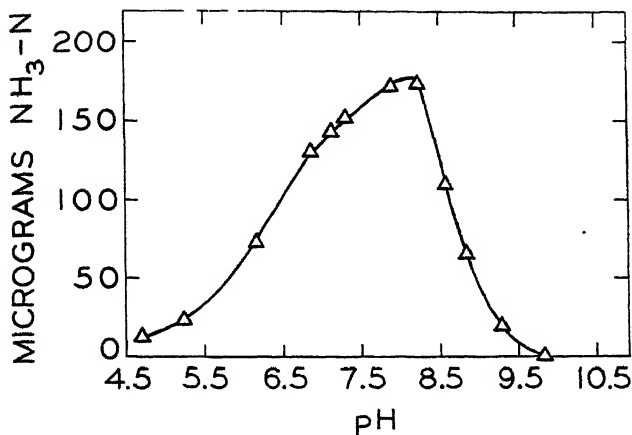


FIG. 1. Relation of pH to hydrolysis of DL-proline amide by purified mushroom preparation. Digests consisted of 1 ml. of preparation  $P_2$  which had been diluted with water to a concentration of 0.12 mg. N/ml., plus 2 ml. of buffer, plus 1 ml. of 0.05  $M$  substrate. Incubation period: 30 min. at 38°. Veronal buffers were used at pH 4.7-7.9; borate buffers were used above pH 7.9. Neither enzyme nor substrate solutions yielded measurable blank values.

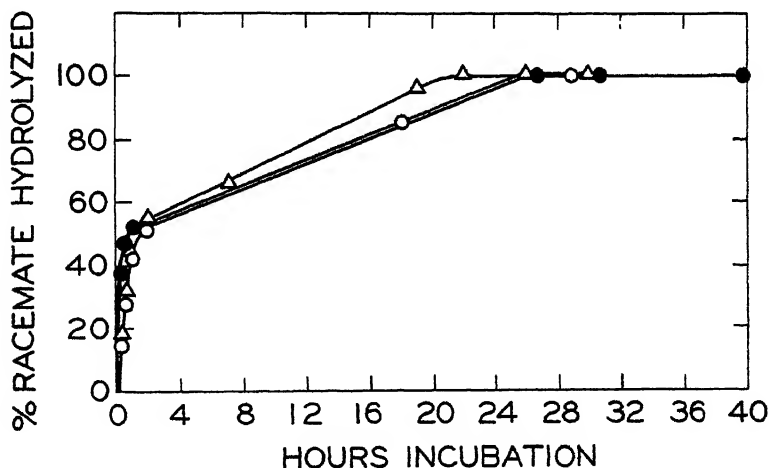


FIG. 2. Hydrolysis of both optical forms of racemic amino acid amides by purified mushroom preparations. Starting molarity of proline amide solution 0.05, of leucine and of alanine amides 0.02. pH of the digests 8.0. Temperature 38°. Enzyme solutions contained 0.7 mg. N/ml. Neither enzyme nor substrate solutions yielded measurable blank values.  $\Delta$  Proline amide,  $\bullet$  alanine amide,  $\circ$  leucine amide.



In order to confirm the analytical data, the proline formed by action of the mushroom preparation on the racemic amide was isolated and its optical rotation determined. The racemic proline amide (28.5 g.) was dissolved in 250 ml. of water, brought to pH 8.0 by addition of acetic acid, and treated with 220 ml. of enzyme fraction P<sub>1</sub>. At the end of 24 hr. incubation, when the carboxyl nitrogen titer indicated that both optical forms had been hydrolyzed (cf. Fig. 2), the digest was brought to pH 5 by addition of acetic acid and the protein filtered through a layer of norite. The filtrate was evaporated *in vacuo* to a low bulk and the proline isolated in the usual manner as the copper salt. The free proline obtained was recrystallized from alcohol. N: calculated, 12.2%; found, 12.2%. The yield of pure proline was 12 g. At a 4% concentration in water, no measurable optical rotation was observed.

### SUMMARY

Homogenates of rat liver, kidney, and intestinal mucosa, or of hog pancreas, possess little or no capacity to hydrolyze proline amide.

In contrast, preparations of mushrooms readily hydrolyze proline amide as well as alanine amide and leucine amide. Both optical forms of the racemic amino acid amides are enzymatically hydrolyzed at the amide bond, one form (presumably the L-) at a very much greater rate than that of the other.

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# Hydrolysis of Proteins Under Pressure

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## INTRODUCTION

In the usual procedures for protein hydrolysis by acids, alkalies, or enzymes, extraneous materials such as salts formed by neutralization, and degradation products of the enzyme are left in the hydrolysate. Use of a gaseous hydrolytic agent would obviate this and hence merits further study.

Sadikov (1) obtained an abiuret product by heating proteins with carbon dioxide under pressure, but presented no experimental data. Komatsen and Okinaka (2) found that a part of casein went into solution under steam pressure obtained by heating with water in sealed tubes, but they did not study the nature of the product. More recently, Lieben (3) reported 20% and 38% hydrolysis by autoclaving a 1% solution of gelatin at 200° and 250°C., respectively.

It has been reported that carbohydrates such as starch (4) or inulin (5) could be hydrolyzed satisfactorily by autoclaving their aqueous solutions under carbon dioxide or steam pressures.

The present report deals with the hydrolysis of casein and gelatin in an aqueous medium and when subjected to elevated temperatures and pressures, with or without carbon dioxide.

## EXPERIMENTAL

The hydrolysis was carried out in two types of autoclaves, one vertical fixed type and the other horizontal rotatory. The autoclaves consisted of thick stainless steel cylinders of 1-l. capacity; to these the lids could be tightened by bolts. They could be heated in electric furnaces to 350°C. and withstood pressures of 200 atm. Gas could be introduced through an inlet tube with a pin valve. An anchor-shaped stirrer rotated by a motor and gear was fitted to the lid of the vertical autoclave. The rotation of the horizontal autoclave about its axis achieved a better mixing,

which was facilitated by putting two or three small stainless steel rods in it. The temperature of the horizontal autoclave could be maintained within  $\pm 2-4^{\circ}\text{C}$ . and of the vertical one,  $\pm 10^{\circ}\text{C}$ .

Commercial Argentine casein (total N, 12.0%) and commercial gelatin (total N, 14.0%) were the proteins used in these experiments. A 5% suspension or solution was employed in each case.

The procedure for hydrolysis consisted essentially in placing the protein suspension or solution in the vessel, closing the lid and introducing carbon dioxide at a pressure of 50–60 atm. The vessel was then placed in the furnace which had been already heated to the desired temperature and the stirring was begun. It took 1–2.5 hr. for the contents of the autoclave to attain the desired temperature of 100–300°C., respectively, and an approximately similar period for cooling down to room temperature after heating was discontinued. This period of preheating and cooling was not taken into account however. The figures for time of hydrolysis presented in the accompanying tables indicate the time for which the charge was maintained at an elevated temperature. The contents were removed next day after allowing carbon dioxide to escape. The vessel was washed and the washings added to the main solution. A small black or brown residue was sometimes left over, which was discarded.

The hydrolysate solutions were acidified to methyl red, boiled for 5 min. and filtered through paper. They were from yellow to deep brown in color, having an acidic bitter taste. They remained without decomposition for 2–3 months under toluene in a refrigerator. Some samples were concentrated under vacuum and then dried at low pressure over silica gel at 70°C., when yellow or brownish hygroscopic solids were obtained.

### *Analysis*

Aliquots of the hydrolysate filtrates were analyzed for total nitrogen by the Kjeldahl method,  $\alpha$ -amino nitrogen by Van Slyke's nitrous acid method, and ammonia nitrogen by A. O. A. C. aeration method (6). The presence of ammonia introduces some error in the determination of amino nitrogen (7), but as no definite relationship was observed between the nitrogen liberated and the concentration of ammonium salts, amino nitrogen values reported here were not corrected for this error. The degree of hydrolysis in per cent was calculated from the ratio of amino nitrogen concentration in the hydrolysate to that of

complete acid hydrolysate of the particular protein. Since ammonia formation is preceded by protein hydrolysis to amino acids and since all of it is not estimated as amino nitrogen, the calculated values for degree of hydrolysis represent only low estimates.

For tryptophan determination, where carried out, solid caustic soda was added to an aliquot of the hydrolysate up to 5 *N* concentration and refluxed in an oil bath at 125°C. for 5 hr. Further procedure was according to Block and Bollings' (8) adaptation of the Millon-Folin procedure. A Klett-Summerson photocolormeter with filter S 42 was used.

Complexity of the hydrolysates was determined by precipitation with 5% trichloroacetic acid according to Evans (9), total nitrogen being estimated in the precipitate.

### DISCUSSION

The results of the experiments with casein using the vertical autoclave (Table I) did not show the same consistency as later experiments with the horizontal autoclave. This was evidently due to the heterogeneous character of the charge and the absence of an efficient means of stirring and regulation of temperature. Solubility of casein improved with increased temperature, most of it going into solution after heating for about 3 hr. at 150–60°C. The degree of hydrolysis was also greater at higher temperatures, and the nitrogen precipitated by 5% trichloro-

TABLE I  
*Effect of Carbonic Acid and Temperature on Autoclave Hydrolysis  
and Tryptophan Content of Casein*

The maximum value for  $\frac{\text{Amino N}}{\text{Total N}}$  after hydrolysis with 6 *N* H<sub>2</sub>SO<sub>4</sub> = 81%.

Expt. no.	CO <sub>2</sub> — Pressure	Tem- perature	Period	Solubility	Extent of hydrolysis	N pptd. by CCl <sub>3</sub> COOH	Tryptophan per 16 g. N <sup>a</sup>
	<i>atm.</i>	<i>°C.</i>	<i>hr.</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>g.</i>
1	50–55	100–120	2	76.3	23.2	14.9	0.66
2	55–60	100–110	8	56.2	35.3	24.0	—
3	55–60	100–110	4	35.3	24.8	—	1.53
4	55–60	150–160	4	92.7	56.8	4.0	0.39
5	55–60	150–160	8	92.3	48.5	1.8	—
6	55–60	150–160	3	95.2	37.6	—	0.74

<sup>a</sup> Tryptophan content of the casein used was 1.57 g./16 g. N.

acetic acid gradually diminished. Tryptophan destruction was considerable above 100°C. and a 4-hr. heating period.

Data reported in Tables II and III were obtained using the horizontal autoclave where temperature regulation and mixing were quite efficient. There was a steady increase in the degree of hydrolysis of gelatin with increasing temperatures (Table II). Values for ammonia nitrogen also increased, particularly at and above 250°C. Thus after 4 hr. of autoclaving at 300°C., nearly 72% of the total nitrogen was in

TABLE II

*Effect of Carbonic Acid and Temperature on Autoclave Hydrolysis of Gelatin*

Blank  $\frac{\text{Amino N}}{\text{Total N}}$  value for 5% gelatin solution = 4.8%.

Maximum value for  $\frac{\text{Amino N}}{\text{Total N}}$  after hydrolysis with 6 N H<sub>2</sub>SO<sub>4</sub> = 71.9%.

Expt no	CO <sub>2</sub> —Pressure	Temperature	Period	Extent of hydrolysis	$\frac{\text{Ammonia N}}{\text{Total N}}$
	atm	°C.	hr	%	%
1	40-50	150	4	15.0	5.4
2	40-50	150	4	15.1	3.4
3	40-50	150	4	14.6	4.5
4	40-50	200	4	54.2	17.0
5	40-50	200	4	54.1	17.6
6	40-50	200	4	60.7	20.7
7	40-50	250	4	77.5	39.5
8	40-50	250	4	81.9	40.0
9	40-50	300	4	85.1	71.9
10	40-50	250	1	77.8	32.2
11	40-50	250	0.5	72.2	28.7
12	No CO <sub>2</sub>	250	0*	60.8	23.9
13	No CO <sub>2</sub>	250	1	73.2	31.2

\* Heating was stopped as soon as the contents attained a temperature of 250°C.

the form of ammonia. The extent of hydrolysis was only slightly lowered by reducing the period of autoclaving from 4 hr. to 1 hr. or half an hour. Most of the hydrolysis seems to have taken place during the period of preheating. Experiments 12 and 13 showed that carbon dioxide itself had very little effect upon the rate of hydrolysis, the more important factor being the temperature.

Essentially similar results were obtained in experiments with casein on the horizontal autoclave (Table III). The degree of hydrolysis

shows a direct relationship to the temperature and was only slightly lowered in the absence of carbon dioxide.

Thus, although a considerable degree of hydrolysis took place when a suspension or solution of a protein in water was autoclaved at elevated temperatures, there was also an appreciable amount of ammonia formed as a result of the decomposition of amino acids. The question whether the ammonia formation can be decreased without seriously lowering the hydrolysis by subjecting the proteins to elevated

TABLE III

*Effect of Carbonic Acid and Temperature on Autoclave Hydrolysis of Casein*

Maximum value for  $\frac{\text{Amino N}}{\text{Total N}}$  after hydrolysis with 6 N  $\text{H}_2\text{SO}_4 = 81\%$ .

Expt no	CO <sub>2</sub> - Pressure	Temperature	Period	Percent of hydrolysis	$\frac{\text{Ammonia N}}{\text{Total N}}$
	atm	°C	hr	%	%
1	55	150	4	23.9	13.9
2	60	200	4	45.0	26.1
3	60	120	4	18.5	10.1
4	60	200	1	33.8	20.7
5	60	200	2	40.0	21.4
6	No CO <sub>2</sub>	150	4	22.0	16.6
7	No CO <sub>2</sub>	200	4	35.7	24.6
8	No CO <sub>2</sub>	250	4	50.9	42.7

temperatures for very short periods could not be investigated owing to the limitations of the apparatus used.

### SUMMARY

When a 5% solution of gelatin in water was autoclaved with carbon dioxide at a pressure of 50–60 atm., the degree of hydrolysis of gelatin during a 4-hr. period increased from 15–85% with increasing temperatures over a range of 150–300°C. Concurrently, there was progressive ammonolysis over the temperature range 150–250°C. and, at a more rapid rate, beyond 250°C.

For hydrolysis of gelatin under pressure, the temperature was the primary factor, the period of autoclaving having considerably less effect. At 250°C., the hydrolysis after half an hour was only slightly less than after 4 hr.

The effect of carbonic acid as a hydrolytic agent seems to be slight. Substantially similar values for protein cleavage were obtained with or without carbon dioxide.

Results obtained with a 5% suspension of casein in water were similar to those with gelatin. Tryptophan was preserved well with a four-hr. heating period at 100–10°C. but there was appreciable loss with higher temperatures and longer periods. Complexity of the product decreased with increasing temperatures.

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# Citric Acid Formation from $C^{14}O_2$ by *Aspergillus niger*<sup>1</sup>

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## INTRODUCTION

Because of the industrial importance of citric acid production by *Aspergillus niger*, speculations concerning the mechanism of its formation have been common. The older hypotheses are of two general types. The first suggests a series of reactions in which the glucose chain is not broken into simpler units but becomes transformed in some way to citric acid. Such hypotheses are considered inadequate since they do not account for citric acid formation from other than 6- and 12-carbon compounds. The second type proposes that glucose is broken into simpler units and that these are synthesized to citric acid; for example, citric acid might arise from pyruvate, or some other 3-carbon compound, via a modified Krebs tricarboxylic acid cycle. Although such schemes can account for the production of citric acid from a variety of compounds, they do not explain the high yields frequently obtained from glucose and sucrose (1).

After Wood and Werkman (2) demonstrated heterotrophic  $C^{14}O_2$ -fixation, a theoretical pathway was opened that would account for these high yields, the key reaction being the utilization of liberated  $C^{14}O_2$ . Qualitative demonstration that  $C^{14}O_2$  from the atmosphere is incorporated into citric acid by *A. niger* has been made by Foster *et al.* (3) using  $C^{11}$  and by El Kerdany (4) using  $C^{13}$ . However, quantitative data as to the extent of such utilization were not provided by these studies. Before final decision as to the significance of  $CO_2$ -fixation in citric acid production can be made, such quantitative studies are essential.

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## EXPERIMENTAL PROCEDURE

*Fermentation*

*Aspergillus niger* strain 72-4, a strain well suited for citric acid production in submerged culture, was used throughout this work (5, 6). The cultures were carried in soil stocks and before use were transferred at least three times on the sporulation medium recommended by Shu and Johnson (6). In the isotopic trials, preformed mycelial pellets were used. Fifty ml. of the fermentation medium of Shu and Johnson (7) in a 500-ml. Erlenmeyer flask was inoculated with a suspension of spores in distilled water. The cultures were incubated at 25°C. on a reciprocating shaker. After 6 days' incubation the pellets were washed twice by centrifugation in nitrogen-free fermentation medium and resuspended in the same medium. The several determinations to be made suggested the use of a modified Warburg type of apparatus. A macro flask (125-ml. capacity) was constructed so that the contents of the center well could be tipped into the side arm. This made it possible to generate labeled  $\text{CO}_2$  at any time without disturbing the system, simply by tipping the acid placed in the well into labeled  $\text{Ba}^{14}\text{CO}_3$  in the side arm. At the end of the run the contents of the flasks were analyzed for total citric acid, activity of the citric acid, and activity of the  $\text{CO}_2$  in the atmosphere.

*Analytical*

Citric acid was estimated by the method of Perlman, Laury, and Johnson (8); it was isolated as the calcium salt. The filtered culture liquor was made just acid to phenolphthalein and then cooled in an ice bath and an excess of ice cold saturated calcium chloride was added to the chilled liquor. After storage overnight in the refrigerator, the solution was filtered, and the calcium citrate was precipitated by heating the solution to boiling. The salt was removed by filtration and washed with water. Samples for degradation studies were purified further by reprecipitation as described by Peterson *et al.* (9). Radioactivity was determined by converting the carbon to  $\text{BaCO}_3$  and counting this by standard methods using an end-window Geiger counter.

*Position of Carbon*

The citric acid was oxidized to  $\text{CO}_2$  by the "wet" oxidation method, trapping the  $\text{CO}_2$  as  $\text{BaCO}_3$  (10). The  $\text{BaCO}_3$  was removed by centrifugation and dehydrated with absolute ethanol. A thick slurry of the precipitate was made in absolute ethanol and dried in aluminum counting dishes. For separating the carboxyl groups, about 100 mg. of labeled calcium citrate was freed of the calcium by treatment with  $\text{H}_2\text{SO}_4$ . The  $\text{CaSO}_4$  was removed by centrifugation and the supernatant liquid transferred to the reaction vessel of a modified "wet carbon" apparatus. In this apparatus the tube normally containing granular zinc was replaced with a trap containing a solution of  $\text{AgNO}_3$  which served to remove any bromine vapors liberated during the reaction. The volume in the reaction vessel was reduced to about 1 ml. and then cooled to 12°C. One ml. of 3%  $\text{KMnO}_4$  at 12°C. was added. The reaction was allowed to proceed at 12°C. for 3 hr. The  $\text{CO}_2$  liberated was trapped by flushing with  $\text{CO}_2$ -free air into  $\text{Ba(OH)}_2$ . This  $\text{CO}_2$  represents the secondary carboxyl group of the citric acid mole-

rule. The solution now contains acetone dicarboxylic acid (11). This compound gives a red color with ferric chloride solution.

After replacing the Ba(OH)<sub>2</sub> trap, 4 ml. of diluted H<sub>2</sub>SO<sub>4</sub> (1:1) and 4 ml. of freshly prepared bromine water were added, and the reaction mixture was allowed to stand at room temperature for 10 min. Two ml. of KBr solution (15 g. KBr in 40 ml. H<sub>2</sub>O) was added, and the mixture heated for 5 min. After cooling, 10 ml. of 5% KMnO<sub>4</sub> was added. The CO<sub>2</sub> liberated was trapped as BaCO<sub>3</sub>. This CO<sub>2</sub> represents the *primary carboxyl* groups. Pentabromoacetone is left in solution (12). This compound is soluble in petroleum ether and gives a yellow color with a mixture of sodium sulfide and dioxane (8).

Separation of the carboxyl groups from the straight-chain portion of the molecule was accomplished by freeing about 250 mg. of calcium citrate of the calcium as already described. The supernate was then transferred to a 300-ml. reaction vessel of the modified "wet carbon" apparatus, and 75 ml. H<sub>2</sub>O, 10 ml. of diluted H<sub>2</sub>SO<sub>4</sub>, and 10 ml. of bromine water were added. The mixture was allowed to stand for 10 min., and after the addition of 5 ml. KBr solution, it was heated for 5 min. On cooling, 25 ml. of 5% KMnO<sub>4</sub> was added, and the liberated CO<sub>2</sub> was collected as BaCO<sub>3</sub>. This sample of BaCO<sub>3</sub> represents the *total carboxyl* groups.

The reaction vessel was cooled in an ice bath, and 40 ml. of ice-cold FeSO<sub>4</sub> solution (20 g. FeSO<sub>4</sub>, 100 ml. H<sub>2</sub>O, and 1 ml. H<sub>2</sub>SO<sub>4</sub>) was added. After thorough shaking, the reaction mixture was placed in a refrigerator for 24 hr. The pentabromoacetone which precipitated was recovered on an asbestos pad in a Gooch crucible and thoroughly washed with ice-cold 1% H<sub>2</sub>SO<sub>4</sub> and then with ice water (13). After drying in a vacuum desiccator, an aliquot was "wet ashed" and the trapped CO<sub>2</sub> counted. This fraction represents the straight-chain portion of the citric acid molecule. A similar degradation was made on another sample by treating the reaction mixture, left after the separation of the individual carboxyl groups, with FeSO<sub>4</sub> solution to obtain the pentabromoacetone (4, 10).

## EXPERIMENTAL RESULTS

It was recognized that the closed system necessary for carrying out the isotopic trials probably would adversely affect the formation of citric acid by the mold. Comparison with control flasks continuously supplied with oxygen demonstrated that both the rate and yield of citric acid decreased rapidly in the closed system. In experiments with tracers, reduction of the time of exposure to the labeled material is, in general, highly desirable to avoid equilibrium conditions. With these two considerations in mind, 4 hr. was selected as the time of exposure even though the quantity of citric acid formed was often too small to be useful for answering the question posed. Three experiments were obtained in which the formation of citric acid, though far from satisfactory, was sufficient to merit estimating the activity of the products.

The detailed manipulations and results from the best of these (in the sense of the quantity of citric acid formed) will be described:

The pellets were prepared as described under *Experimental Procedure*, and after resuspending in the nitrogen-free fermentation medium so that their concentration was the same as before washing, they were shaken for 2 hr. at room temperature while a slow stream of oxygen was bubbled through. Ten-ml. portions of this suspension were transferred to the macro Warburg flasks. In one flask 0.5 ml. of 1 *N* HCl was placed

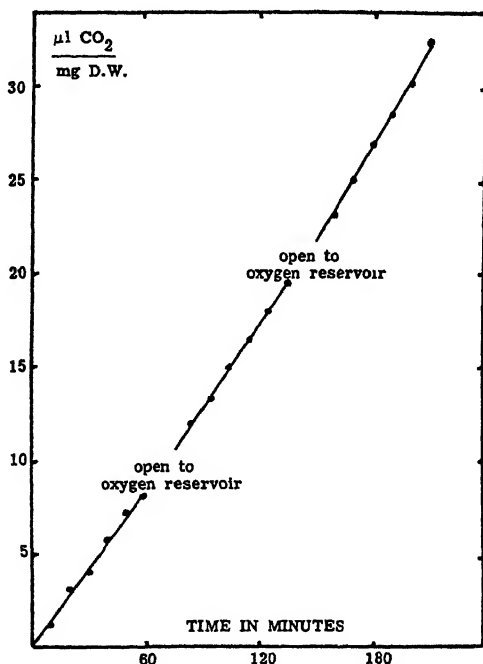


FIG. 1. Production of CO<sub>2</sub> by *Aspergillus niger* in a closed system

in the center well and BaC<sup>14</sup>O<sub>3</sub> in the arm; in another, 0.5 ml. of 20% KOH was placed in the well. Oxygen was passed through each flask in turn at a rate of about 2 bubbles/sec. for 1 min.; the system was closed and the C<sup>14</sup>O<sub>2</sub> liberated. The experiment was continued for 4 hr.; during this period, manometric readings were taken for three 1-hr. periods; between these periods the flasks were connected to an oxygen

well by a manifold. From the manometric data of the two flasks the CO<sub>2</sub> production can be calculated (14).

Analysis before and after the exposure to the labeled CO<sub>2</sub> showed that 40.6  $\mu$ moles of citric acid had been formed; 264  $\mu$ moles of metabolic CO<sub>2</sub> was produced, and 12.19  $\mu$ moles of CO<sub>2</sub> was added as the labeled BaC<sup>14</sup>O<sub>3</sub>. Since the production of metabolic CO<sub>2</sub> was essentially linear (Fig. 1), the dilution factor of the labeled CO<sub>2</sub> was taken as equal to one-half the total CO<sub>2</sub> liberated (metabolic plus labeled) divided by the labeled: 138.09/12.19, or 11.3. From the specific activity and the quantity of BaC<sup>14</sup>O<sub>3</sub> used, it was calculated that  $399 \times 10^3$  counts/min. was added to the flask as labeled CO<sub>2</sub>. Isotopic analysis of the citric acid revealed a specific activity of 1030 counts/min.; the total citric acid was 36.8 mg.; therefore, the total citric acid accounted for  $38 \times 10^3$  counts, or 9.6% of the original activity of the CO<sub>2</sub> supplied. The pickup of CO<sub>2</sub> can be estimated from the CO<sub>2</sub> supplied in  $\mu$ moles times the dilution factor times the per cent C<sup>14</sup> in the citric acid:  $12.19 \times 11.3 \times 0.096 = 13.2$   $\mu$ moles. Since citric acid contains 6 atoms of carbon, a total of  $6 \times 40.6 = 243.6$   $\mu$ moles of carbon was represented in the citric acid formed; accordingly, the carbon arising from CO<sub>2</sub> would be  $13.2/243.6$  or 5.5% of the total. In two other experiments, the values obtained were 2.6% and 3.6%. The following table summarizes these data:

TABLE I  
1. Fermentation Data  
(In  $\mu$ moles)

Citric acid		Carbon dioxide		Dilution factor
Initial	Formed	Output	Supplied	
192.7	40.6	264	12.19	11.3

2. Isotopic Data

(Total count $\times 10^{-3}$ )		%-C <sup>14</sup> O <sub>2</sub>
Original C <sup>14</sup> O <sub>2</sub>	Citric acid	In citric acid
399	38	9.6

3. Calculations

CO<sub>2</sub> pickup;  $0.096 \times 12.19 \times 11.3 = 13.2$   $\mu$ moles.

CO<sub>2</sub>-C in citric acid;  $13.2/(40.6 \times 6) = 0.055$  (or 5.5%).

The degradation studies showed that the CO<sub>2</sub>-C appeared only in the carboxyl groups of the citric acid molecule. The labeled carbon was

almost equally distributed between the primary and the secondary carboxyl groups. The data obtained from two degradations are shown in Table II

TABLE II  
*Degradation of Labeled Citric Acid*

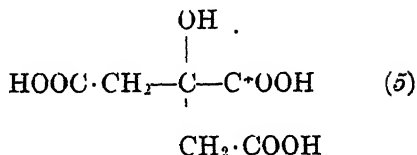
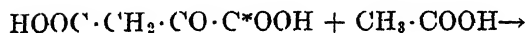
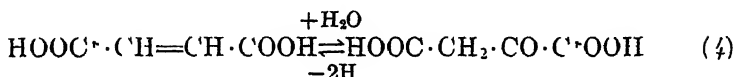
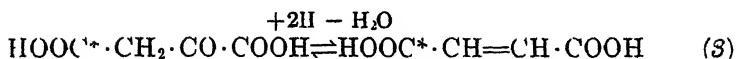
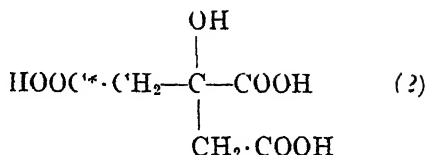
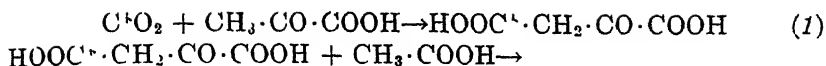
Fraction	Specific activity of BaC ( )	
	Exp 1 ct /mg /sec	Exp 2 ct /mg /sec
Total carbon	3.22	4.54
COOH-carbon	5.76	—
Straight-chain carbon	0	0
Primary COOH-carbon	5.84	7.87
Secondary COOH-carbon	5.35	7.09

Theoretically the carboxyl-carbon atoms should have twice the specific activity of the total carbon atoms of the molecule. In these experiments, however, this ratio was not quite achieved. It should be noted, however, that the straight-chain portion of the molecule possessed no activity.

An experiment was made to determine if citric acid was being utilized during the time that sucrose was fermented. To each of six shake cultures of sucrose medium was added from 6 to 8 mg. of labeled citric acid. One set of three cultures served as heat-killed controls; CO<sub>2</sub>-free air was passed through each set of these cultures and then through NaOH traps. The whole apparatus was assembled on a reciprocating shaker at 25°C. and was incubated for 3 days. At the end of the run, the CO<sub>2</sub> trapped in the NaOH was precipitated by the addition of BaCl<sub>2</sub>. The BaCO<sub>3</sub> was counted as previously described. The data indicated that citric acid was not utilized in the presence of sucrose. This was in agreement with the observation that the citric acid in a culture does not begin to disappear until the sucrose has been utilized.

## DISCUSSION

Formation of citric acid through assimilation of CO<sub>2</sub> is now generally assumed to proceed by a mechanism such that 1 mole of CO<sub>2</sub> is fixed per mole of citric acid. For example, the following scheme, which can be taken as a representative of these proposals, would account for the observed position of the labeled carbon:



Reactions (1) and (2) provide a mechanism for entrance of C<sup>14</sup>O<sub>2</sub> that would appear in the primary carboxyl groups of the citric acid. It should be noted that citric acid labeled in either one of the terminal carboxyls would apparently be labeled in both terminal groups when subjected to chemical degradation. Reactions (3), (4), and (5) illustrate a process whereby the labeled carbon could enter the secondary carboxyl group. As these two sets of reactions need not proceed at the same rate, such a scheme could explain why El Kerdany (4), using a different strain of the organism, found the labeled carbon predominantly in the terminal carboxyl groups, whereas we found the label equally distributed among the carboxyl groups.

The report of Weinhouse and Lewis (15) that citric acid formation by *A. niger* occurs by way of a C<sub>2</sub>-C<sub>4</sub> condensation, and that acetic acid is an intermediate of glucose and sucrose oxidation, lends support to the scheme presented above. Also Stern and Ochoa (16) have obtained an enzyme preparation from pigeon liver that catalyzes the

synthesis of citrate from oxalacetate and acetate in the presence of adenosine triphosphate and coenzyme A.

Through reactions (1) to (5) or some analogous mechanism the theoretical yield for the incorporation of  $\text{CO}_2$  would be 16.6%. In these trials we have observed from 2.6–5.5%, or 16–33% of the theoretical. Admittedly values closer to 16.6% are desirable, but because of the rather unsatisfactory environmental conditions for production of citric acid that must be used in making the test, perhaps these are about as good as one may reasonably expect. It might be possible to obtain more satisfactory values by refinements in technique such as methods for estimating citric acid, the dilution factor, and the like, but the primary objection to the experimental conditions—carrying out the fermentation in a closed system—is more difficult to resolve. In spite of these difficulties the observed uptake appears to justify the conclusion that  $\text{CO}_2$  can take part in the formation of citric acid by this organism in a manner that is quantitatively significant.

#### SUMMARY

Isotopic studies with  $\text{C}^{14}$  on the fixation of  $\text{CO}_2$  by *Aspergillus niger* demonstrate that  $\text{CO}_2$  is fixed by the mold and that the  $\text{CO}_2\text{-C}$  appears in the citric acid produced. Chemical degradation of the labeled citric acid shows that the carbon label appears exclusively in the carboxyl groups of the citric acid molecule and that the  $\text{C}^{14}$  is almost equally distributed between the primary and secondary carboxyl groups. Experiments designed to investigate the magnitude of the fixation reaction demonstrate that  $\text{CO}_2$  can be an important intermediate in the formation of citric acid by *A. niger*.

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# Effects of Borate and Other Ions on the Alkaline Phosphatase of Bovine Milk and Intestinal Mucosa

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## INTRODUCTION

The inhibitory effect of borate on alkaline phosphatase has frequently been noted (1-6), but no quantitative studies have been reported. Such a study appeared to be desirable because borate reacts with riboflavin (7), pyridoxine (8), muscle adenylic acid (9), and polysaccharides (5, 10, 11), and inhibition of alkaline phosphatase might be due to interaction with some such component of the enzyme.

Preliminary to the study of the effect of the borate, investigations were made to find a suitable buffer in which to perform the enzymatic reaction. Ethanolamine and its hydrochloride were chosen as being most satisfactory. The relative inhibition of the phosphatase by certain anions other than borate was also determined.

## METHODS

### *Preparation of the Alkaline Phosphatase of Milk*

Unpasteurized cow's milk was treated with rennet, and the coagulum of casein was removed by filtering through cheesecloth. Solid ammonium sulfate was added to the filtrate until the concentration was 2.3 *M*. The precipitate was redissolved in water to give a protein concentration of about 3%, and digested with trypsin until the precipitation with trichloroacetic acid had decreased about two-thirds. This digest was mixed with an equal volume of 1.5 *M*  $\text{Na}_2\text{SO}_4$  and 0.2 volume of toluene, shaken for several hours, and filtered. An equal volume of 1.5 *M*  $\text{Na}_2\text{SO}_4$  was added to the filtrate, and the precipitate was discarded. Twenty g. of ammonium sulfate was added to each 100 ml. of solution. The precipitate was dissolved, dialyzed to reduce the salt concentration, and kept at 7°C., pH 7.5 to 8.5, with chloroform present. The solution

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contained 0.25% protein (Biuret), and when assayed in a dilution of 1:25, gave a reading of 16.3. The activity decreased about 10% in 2 weeks.

This method resulted in a purification of about 60-fold and removed a lipid fraction which always accompanied the enzyme in other methods of purification tried. For maximum stability during the purification, the pH was kept in the range 7.5 to 8.5.

### *Preparation of the Alkaline Phosphatase of Intestinal Mucosa*

Preparation of this material from the intestinal mucosa of the calf has been described (12). A stock solution, prepared by dissolving 10 mg. in 99 ml. of water and 1.0 ml. of 0.1 *M* sodium veronal (final pH about 8.6) and adding 0.5 ml. of chloroform, had not changed in activity in 6 months at 7°. One ml. of a 1:25 dilution gave a reading of 29.0 in the assay procedure.

### *Assay of Alkaline Phosphatase*

The substrate used was sodium phenylphosphate in a concentration of 0.00075 *M*. The phenol released by the enzyme was determined with the reagent of Folin and Ciocalteu (13). In the assay, 0.9 ml. of 0.01 *M* substrate is diluted with 8.1 ml. of 0.1 *M* buffer, and 0.2 ml. of 0.15 *M*  $MgCl_2$  and 1.8 ml. of water is added. The temperature of this mixture is raised to 37°, and 1.0 ml. of enzyme is added. After the enzyme has acted for 5 min., 4.0 ml. of the Folin reagent is added, stopping the reaction. The phenol color is developed by adding 6.4 ml. of 1.9 *M*  $Na_2CO_3$  and holding at 37° for 30 min. The blue colors are read on the logarithmic scale in a photometer with a No. 650 filter. With the milk enzyme, an inactive-enzyme blank is used; with the mucosa enzyme, a reagent blank gives the total extraneous color. The results are expressed as photometer readings or as moles of phenol liberated. When the 0.1 *M* ethanolamine buffer is employed, a reading of 14.3 is equivalent to  $1.0 \times 10^{-7}$  moles of phenol; in 0.05 *M* ethanolamine buffer, the reading is 13.0; with all the other buffers and anions studied, a reading of 12.0 is obtained with the same amount of phenol. The data in almost every case represent duplicate analyses. The hydrolysis of the substrate was less than 5%, except in the few experiments with extremely dilute substrate shown in Fig. 4.

## RESULTS

The assay values given under *Methods* were obtained at pH 9.67 with 0.1 *M* (0.068 *M* final concentration) ethanolamine-HCl buffer (pK of ethanolamine is 9.5). Sodium veronal, used in initial studies, gave variable final pH values. Veronal and several other buffers used for the assay of alkaline phosphatase were investigated in respect to variation in enzyme activity with change of pH. Figure 1 gives the results. One ml. of the enzyme diluted 1:25 was used, except in the experiment with mucosa enzyme in borate, in which the dilution was 5:25. All data are expressed on a 1:25 basis since experiments with several concentrations

of the enzyme showed that the activity was proportional to the concentration. The data obtained with the mucosa and the milk enzymes are similar, but the optima for the milk enzyme lie at slightly higher pH values, and the order of activity in the veronal and ethanolamine buffers is reversed. Consideration of the effect on the enzyme action, and pH optimum in relation to maximum buffering action, led to the main use of the ethanolamine buffer for subsequent studies.

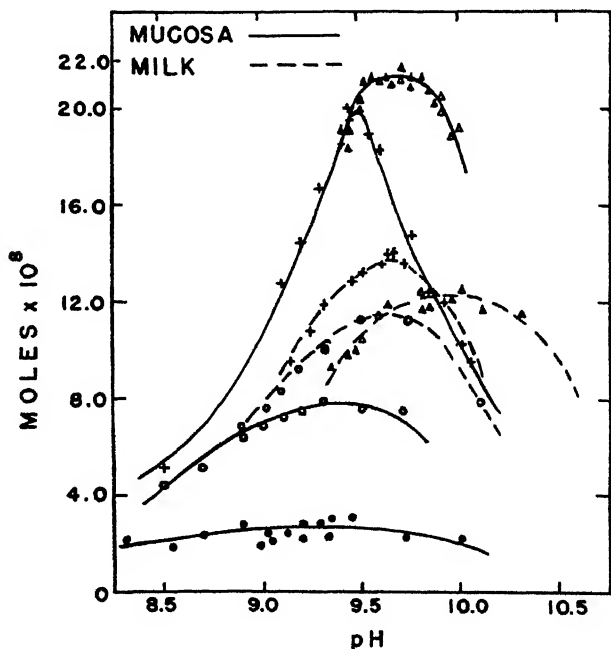


FIG. 1. Activity, in moles of phenol liberated, of the alkaline phosphatase of bovine milk and intestinal mucosa in relation to pH in various buffers.  $\Delta$ , 0.068 *M* ethanolamine-HCl; +, 0.068 *M* sodium veronal-HCl, O, 0.017 *M* sodium tetraborate-HCl, ●, 0.068 *M* sodium tetraborate-HCl.

The effect of borate (this term in every case refers to sodium tetraborate) on the mucosa enzyme in ethanolamine (0.068 *M*, pH 9.67) and carbonate (0.068 *M*, pH 9.37) buffers was investigated. The borate replaced the water in the assay method; final pH values were checked. Figure 2 shows the data obtained. Carbonate was inhibitory to the mucosa phosphatase (Table I), but this does not show in the figure, for

the same base line was chosen as for the ethanolamine buffer. From the data, it can be seen that 0.011 *M* tetraborate caused 50% inhibition (ordinate = 6.9) of the mucosa enzyme in carbonate; extrapolation of the data shows that 0.019 *M* would be required to give 50% inhibition in ethanolamine. This inhibitory concentration of borate is about the same as that calculated from the activity in borate buffers (Fig. 1) compared with the activity in veronal and ethanolamine. The nonlinear inhibition curve for borate in carbonate, which indicates that in carbonate buffer borate is less inhibitory in dilute solution than in more concentrated solutions, suggests that the borate and carbonate might be competing for the same sites on the enzyme. This was another reason for the choice of the ethanolamine buffer.

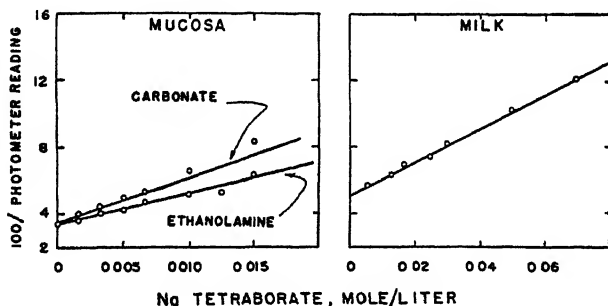


FIG. 2. Effects of borate on mucosa phosphatase in carbonate and ethanolamine buffers, and the effects of borate buffers on milk phosphatase.

Study of the effect of borate on the milk phosphatase showed that this enzyme was much less inhibited than the mucosa enzyme. Since adequate buffering was provided by the concentrations of borate used, the ethanolamine was omitted. Inhibition by borate was of the same magnitude, with or without ethanolamine. Figure 2 shows these data. The concentration of tetraborate giving 50% inhibition (ordinate = 10.0) was 0.050 *M*.

The effects of 0.005 and 0.01 *M* tetraborate on the mucosa phosphatase in 0.071 *M* ethanolamine at pH 9.6 with several concentrations of substrate ( $1.53\text{--}11.3 \times 10^{-4}$  *M*) were also studied. In Fig. 3, the reciprocals of the reaction velocities ( $1/V$ ;  $V$  = moles/5 min.) are plotted against the reciprocals of the concentrations of substrate ( $1/C$ ). Also presented are the data for the reaction in 0.035 and 0.071 *M* ethanol-

amine with no borate present. On the basis of the analysis of enzyme inhibitions given by Lineweaver and Burk (14), and recently by Harmon and Niemann (15), the intercept of the curve on the abscissa equals  $1/V_{\max}$ , where  $V_{\max}$  is the maximum activity with excess of substrate. The slope of the curve equals  $K_s/V_{\max}$ , where  $K_s$  is the constant for the enzyme-substrate dissociation, and numerically is the concentration of substrate giving one-half the maximum activity. In competitive inhibition, the slope increases and with it the apparent  $K_s$ , with no change in the intercept. It can be concluded that the borate

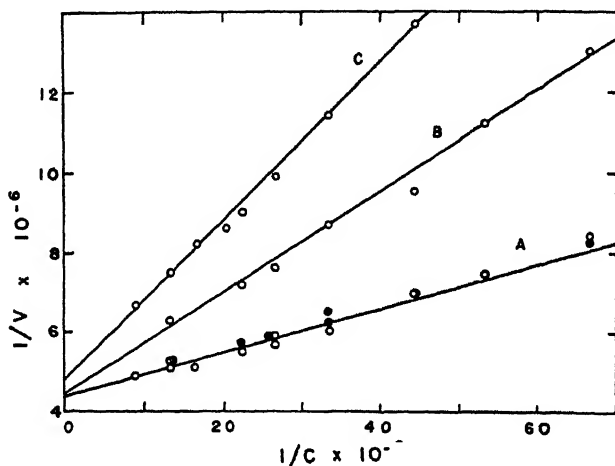


FIG. 3. Effect of borate on mucosa phosphatase (1:25) in relation to concentration of substrate. Velocity ( $V$ ) is expressed in moles/5 min.; concentration of substrate ( $C$ ) is in moles/l. A, no borate:  $\circ$ , 0.035  $M$  ethanolamine;  $\bullet$ , 0.071  $M$  ethanolamine; B, 0.005  $M$  tetraborate; C, 0.01  $M$  tetraborate.

inhibition is of this type, inhibition being greatest with low concentrations of substrate. From the data with no borate, the value of  $V_{\max}$  is  $2.27 \times 10^{-7}$  moles, and the enzyme-substrate dissociation constant  $K_s$  is 0.00013 mole/l. There is a slight shift of the  $1/V$  intercept in the presence of borate, the respective  $V_{\max}$  for 0.005 and 0.01  $M$  tetraborate becoming  $2.22 \times 10^{-7}$  and  $2.08 \times 10^{-7}$  moles; the apparent  $K_s$  values are 0.00028 and 0.00042 mole/l. The linear extrapolation of these  $K_s$  values to zero borate concentration gives a value of 0.00015 mole, in good agreement with that found in the borate-free solutions.

The shift of the intercept on the  $1/V$  axis is evidence for a slight non-competitive inhibition. Jacobsen (16) has reported that the inhibition of alkaline phosphatase by phosphate, although largely competitive, is in part noncompetitive.

Since the results for both concentrations of ethanolamine fall on the same straight line, there is no inhibition of either the competitive or noncompetitive type when the concentration of this buffer is increased.

A similar analysis of the milk phosphatase was made in borate buffers at pH 9.6 (Fig. 4). The phosphatase was diluted 2:25 for these experiments; the same  $K_s$  values were obtained with a 1:25 dilution of the phosphatase. The following data were obtained for 0.035, 0.021, and 0.014  $M$  borate.  $V_{max}$  values:  $2.56$ ,  $2.90$ , and  $3.13 \times 10^{-7}$  moles,

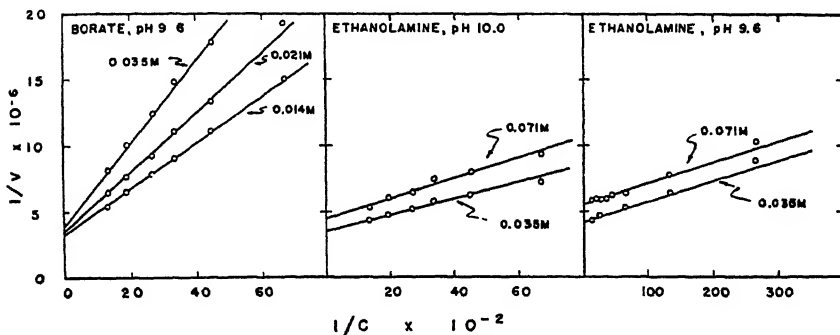


FIG. 4. Effects of borate and ethanolamine on milk phosphatase (2:25) in relation to concentration of substrate. Velocities ( $V$ ) are expressed in moles/5 min.; concentration of substrate ( $C$ ) in mole/l.

respectively; apparent  $K_s$  values: 0.00082, 0.00064, and 0.00053 mole/l, respectively. The value of  $K_s$  from an extrapolation of the apparent values to zero borate concentration is 0.00035 mole/l.

The constant  $K_i$  for the dissociation of the enzyme-inhibitor complex was calculated from the borate inhibition substrate concentration data by the procedure described by Harmon and Niemann (15). For the mucosa phosphatase,  $K_i$  is 0.0036 mole/l; for the milk phosphatase, 0.012 mole/l.

The milk enzyme was also studied at several concentrations of substrate in 0.071 and 0.035  $M$  ethanolamine at pH 9.6 and 10.0, respectively. These data are included in Fig. 4. It is apparent that the milk enzyme is inhibited by ethanolamine and that it is a noncompetitive

inhibition, that is, the  $K_i$  values are essentially the same for the two concentrations of ethanolamine. With the 0.00075  $M$  substrate at both pH values, the inhibition produced by doubling the ethanolamine concentration is 20%. Calculations like those employed with the data in Fig. 2 indicate that the enzyme would be inhibited 50% at concentrations of 0.073 and 0.105  $M$  ethanolamine at pH 9.6 and 10.0, respectively.

In noncompetitive inhibition,  $1/V (1 + i/K_i) = 1/V_i$ , where  $V_i$  is the maximum velocity in the presence of inhibitor, and the inhibitor-

TABLE I  
*Inhibition of Alkaline Phosphatase by Certain Ions*

Ions	Concentrations tested	Concentrations giving 50% inhibition <sup>a</sup>	
		Mucosa enzyme	Milk enzyme
Tetraborate	<i>mole/l</i> 0.0015-0.011 (mucosa) .0125-.100 (milk)	<i>mole/l</i> 0.019	<i>mole/l.</i> 0.052
Phosphate	.004 and .008	.0013	.0108
Pyrophosphate	.004 and .008	.0013	.0120
Carbonate	.04 and .08	.21	—
Arsenate	.0004 and .0008	.00013	.00071
Caodylate	.02 and .04	.095	—
Ethanolamine	.035 and .071	No inhibition	.09

<sup>a</sup> Determined from graphs of the type used in Fig. 2.

enzyme constant  $K_i$  is  $(1/V) (i)/(1/V_i - 1/V)$ . From a plot of  $1/V_i$ , the intercept in the presence of inhibitor, the value of the intercept when no inhibitor is present,  $1/V$ , can be found by extrapolation. By this means,  $K_i$  values were calculated for inhibition by ethanolamine of 0.071 mole/l. at pH 9.6; 0.085 at pH 10.0.

At pH 10.0, the optimum pH for the milk enzyme in ethanolamine, the  $K_i$  is 0.00020 mole/l., about the same as the  $K_i$  in borate (extrapolated to borate = 0) at pH 9.6, the optimum in borate. There is a great dependence of the  $K_i$  on pH, for at pH 9.6 in ethanolamine it is sixfold smaller than at pH 10, that is, 0.000034 mole/l. Milk and the mucosa enzymes have about the same  $K_i$  values at their pH optima (pH 10.0

and 9.6, respectively) in ethanolamine, but when compared at the same pH (9.6) they differ fourfold.

The effects on alkaline phosphatase of the anions phosphate, pyrophosphate, carbonate, and arsenate have frequently been studied. Table I gives the inhibition of these anions on the phosphatases used in the present studies for comparison with the inhibition by tetraborate.

### DISCUSSION

Both milk and mucosa phosphatases are inhibited competitively by sodium tetraborate. Since both phosphate (16) and carbonate (17) also inhibit alkaline phosphatase competitively, it is likely that the borate inhibition is of a similar anionic type rather than due to its ability to react with the polyhydroxy compounds mentioned in the introduction. It is of interest that borate has been found inhibitory to the enzymes urease (18), arginase (19), and pepsin (20). However, none of these contains riboflavin, pyridoxine, or adenylic acid, and it is probable that this inhibition is of the competitive anionic type, since urease, for example, is inhibited competitively by phosphate (15). It may be significant that with arginase, which is activated by manganese, the inhibition by borate is greater than that by phosphate, which is the reverse of that obtained with the magnesium-activated alkaline phosphatase. Tetraborate (0.033 *M*) does not inhibit the riboflavin-containing xanthine oxidase from milk (21), but it would be of interest to see borate tested on other enzymes containing riboflavin, as well as pyridoxine and adenylic acid.

Published enzyme-substrate constants ( $K_s$ ) are difficult to compare because of the influence of pH, buffers, and the kind of substrate. However, the values reported here are of the same magnitude as those reported by others for the hydrolysis of phenyl phosphate. Folley and Kay (22) obtained a value of 0.0006 with the phosphatase of mammary tissue in glycine buffer at pH 10.0. They observed the decrease in  $K_s$  with decrease in pH. The great effect of pH on  $K_s$  was observed by Jacobsen (16) with kidney phosphatase. Schmidt and Thannhauser (23) using intestinal phosphatase at a pH of 9.3 in veronal buffer obtained a value of 0.000072 for  $K_s$ . This value is lower than that obtained in the present studies, but this is to be expected because of the lower pH.

Inhibition of milk phosphatase by ethanolamine is of the noncom-



petitive type, which is the same type shown by the ammonium ion (17). Inhibition of alkaline phosphatase by glycine is also predominantly of the noncompetitive type (21).

The effects of different buffers on the pII optima of kidney (rat) alkaline phosphatase when acting on glycerophosphate have recently been reported (6). The data parallel those of the present study, in that glycine and  $\text{NH}_4\text{OH}$  buffers, which may be compared with ethanolamine, give the highest pII optima, next come veronal, and then borate. The results are comparable too in the broad pII optima for  $\text{NH}_4\text{OH}$ , ethanolamine, and borate and the sharp pII optimum for veronal. The effects of the buffers on the activity are also of about the same general magnitude.

Ethanolamine is an excellent buffer for alkaline phosphatase assays for the reasons mentioned earlier. Further, phosphatase in it has a broad pH optimum, which is desirable. Experiments at pII 9.6 are at the optimal pH for the mucosa phosphatase and only slightly below the optimum for the milk phosphatase; buffering is maximal, and in the case of milk enzyme, because of the low  $K_i$  value, low concentrations of substrate are adequate.

A number of means have been tried to reduce the borate inhibition of mucosa phosphatase to that shown by the milk phosphatase. Heat-inactivated milk enzyme, acid-inactivated mucosa enzyme, alanine, and magnesium were added or varied without changing the inhibition. The phosphatase in whey showed the same inhibition as did the purified phosphatase. It is probable that the difference in behavior of the two enzymes is not due to the presence of a second admixed component. However, it is possible that a second component may have reacted with the one enzyme to give the other. Both enzymes give broad pII optima with borate; both give sharp optima with veronal and both have about the same  $K_i$  values at their optimal pII in ethanolamine.

Some of the data, however, suggest that milk and mucosa phosphatases are different enzymes. The milk enzyme has a higher pII optimum than the mucosa enzyme; at pH 9.6 it has a much lower  $K_i$  value, and it is less inhibited by anions and more inhibited by cations (ethanolamine). Various means have been tried for distinguishing several alkaline phosphatases. Further investigation with phosphatases from different tissues might show that the comparative effects of anions and cations would provide a means for distinguishing two types. Data

recently reported by Bodansky (25) conform with this suggestion. The cationic amino acids histidine and lysine were about three times more inhibitory to bone and kidney phosphatases than they were to intestinal mucosa phosphatase, whereas the anionic glutamic acid was about three times more inhibitory to the mucosa phosphatase than to the other phosphatases. On this basis, the milk phosphatase would be classified with kidney and bone phosphatases. Folley and Kay (22) have noted the close resemblance of kidney and mammary phosphatases on other points.

### SUMMARY

Alkaline phosphatases from bovine milk and intestinal mucosa are inhibited competitively by borate, probably by an ionic effect. Milk phosphatase is inhibited noncompetitively by ethanolamine. Enzyme-substrate dissociation constants ( $K_s$ ) and enzyme-inhibitor constants ( $K_i$ ) have been calculated.

Differences in pH optima, the relative inhibition by borate and ethanolamine, and  $K_i$  values indicate that the phosphatase activity of milk and mucosa represent two different enzymes. It is suggested that the relative inhibition by anions and cations might distinguish intestinal phosphatase from milk, kidney, and bone phosphatases.

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# Enzyme Studies on White Blood Cells.

## I. Glycogen Degradation<sup>1</sup>

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### INTRODUCTION

In a previous paper (1) it was shown that the polymorphonuclear leucocyte is the only representative of the white blood cells in the peripheral blood which contains glycogen. Neither plasma, nor red blood cells, nor blast cells contain this carbohydrate. It is still controversial whether or not it is present in lymphocytes and blood platelets. By histochemical methods Gibb and Stowell (2) demonstrated it in small amounts in platelets as well as in lymphocytes, in contrast to Wislocki and Dempsey's (3) previous studies of this problem. From our chemical studies on leukemic blood with one predominant cell type, the conclusion was reached that glycogen is not present in lymphocytes (1). As far as the blood platelets are concerned, some evidence suggests that the reducing substances found in hydrolysates of platelets originate from ribonucleic acid rather than from glycogen, at least in human blood. Furthermore, it was demonstrated (4) that the glycogen in white blood cells is rapidly degraded, even if the cell suspension is kept at refrigerator temperature under sterile conditions.

The present study is concerned with the nature of the enzyme system involved in the process of glycogen metabolism in the white blood cell. Except for histochemical investigations, few chemical studies have been carried out to elucidate the question. Since Levene and Meyer's (5) classic paper on the conversion of glucose to lactic acid by white blood cells, numerous studies on glycolysis of added glucose in blood and its

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constituents have been undertaken. They are thoroughly reviewed by E. Lundsgaard (6). Willstätter and Rohdewald (7) found that in the presence of intact leucocytes, glucose is converted to glycogen, and the glycogen is then degraded to lactic acid. The first of these two reactions has a higher velocity than the latter. At a pII of 6.6 they were able to demonstrate glycogen synthesis in white blood cells of the horse. There is no indication that there is direct glycolysis in blood or in a suspension of isolated white blood cells. Glycogen is always the intermediary between glucose added and lactic acid formed.

No studies on intact white blood cells have been made dealing with glycogen degradation and search for intermediary products between glycogen and lactic acid without the addition of glucose. It would be interesting to investigate the physiology of a tissue such as the white blood cells which contain about the same percentage of glycogen as the striated muscle.<sup>3</sup> One of the difficulties is the collection of white blood cells in sufficient amounts, and in such a state of purity that the results are not obscured by the presence of other blood constituents. The greatest problem is an almost unavoidable admixture of erythrocytes with the white blood cells isolated from whole blood. In addition, there are always blood platelets present in suspensions of leucocytes. The material has to be worked up quickly, in order that there will be a minimum of glycogen breakdown prior to the actual experiment. None of the methods of isolation so far available has met all of these requirements.

All data reported in this paper are derived from experiments on cell suspensions. Because of the admixture of other cell material, experiments on extracts would not show the enzymatic activity of the leucocytes exclusively. While from other tissues, such as liver or muscle, active aqueous extracts can be prepared, extracts made from leucocytes by crushing the cells in water with sea sand were ineffective. Using cell suspensions and combining the results obtained by control experiments on the contaminants which can be recovered as homogeneous cell material, is so far the most appropriate procedure.

The main purpose of our study was to determine whether or not the glycogen metabolism in the white blood cells follows the same mech-

<sup>3</sup> After this paper was completed, an article of Rohdewald (8) came to our attention. She showed a decrease in inorganic phosphorus in the reaction between autolyzed horse leucocytes or their glycerol extract and glycogen or starch.

anism as that in liver and muscle, which has recently been elucidated by the fundamental work of Cori *et al.* (9).

## EXPERIMENTAL METHODS

### *Collection of White Blood Cells*

There are different techniques available to isolate white blood cells from whole blood. Isolation in Cushman tubes which were used in our previous investigations (1, 4) did not yield sufficient quantities for enzymatic studies. The advantage is the simplicity and quickness of isolating viable cells. The disadvantage is the relatively large number of red blood cells admixed. The number of platelets varies from case to case.

Another method of isolation which was applied in many of our experiments is the flotation method of Vallee, Hughes, and Gibson (10). The separation of the red from the white blood cells is not always quite successful, and there is some admixture of red blood cells with the white blood cells. The blood platelets float together with the white blood cells. The quantitative yield of leucocytes is satisfactory.

A modification of the technique of Vallee, Hughes, and Gibson was described by Spear (11), using gum acacia instead of albumin. For our particular study the use of gum acacia is contraindicated, since the presence of this agent interferes with the subsequent glycogen determination in the white blood cells.

Because large amounts of leucocytes, within the range of several billions, were needed for our experiments, we finally resorted to citrated horse blood. This can easily be obtained in quantities of several liters. The red blood cells settle quickly on standing, the white blood cells remain suspended in the plasma and can be recovered by centrifuging. Red blood cells in varying amounts and blood platelets are always present in the suspension. The ratio of red blood to white blood cells is about 3:2 under favorable conditions; occasionally it was only 6:1. In most of our experiments the leucocytes were collected by this technique. We had to give up the attempt at purifying leucocytes entirely from their two admixtures. To separate the white blood cells completely from erythrocytes and platelets would mean repeated washing and fractionated centrifuging which would entail too much delay.

Recently an improved technique of separating white blood cells from whole blood was described by Minor and Burnett (12), and modified by Buckley, Powell, and Gibson (13). Addition of fibrinogen (Fraction I) accelerates the sedimentation rate of the red blood cells to such an extent that the white blood cells are kept in suspension in the plasma and can be isolated by centrifuging. Buckley, Powell, and Gibson (13) have shown that 80% of the white blood cells can be recovered by this method.

This technique should be successful on horse blood, the red blood cells of which settle rather quickly even without the addition of fibrinogen. If Fraction I is added, the admixture of red blood cells may be reduced to a negligible minimum. The blood platelets have to be carried along. The method is suitable for collecting large amounts of white blood cells. So far our experience with the technique of Buckley, Powell, and Gibson is limited, since most of our experiments were completed when the method was published. It will receive careful consideration in subsequent studies.

In all the procedures mentioned above, the white blood cell suspension represents a mixture of the different types of leucocytes, *i.e.*, polymorphonuclear leucocytes, lymphocytes, and monocytes. A suspension containing only granulated white blood cells can be obtained by de Haan's (14) method. Injection of a sterile physiological sodium chloride solution into the abdomen of a rabbit produces an exudate free from red blood cells, lymphocytes, and blood platelets. Although this method would satisfy most requirements, the quantitative yield is rather poor. Not more than six to seven million granulated cells can be recovered in one experiment. In our previous studies we used the method successfully for some of the experiments. Also an attempt was made at collecting homogeneous white blood cells from sterile abscesses produced by injecting monolate under the skin of the rabbit. However, the pus cells showed marked signs of disintegration owing to the induration and destruction at the site of the injection.

Since the granulated polymorphonuclear white blood cell is considered the sole carrier of glycogen, it is permissible to use mixed white blood cell layers and calculate the results after a differential count of the white blood cells, if absolute values are required.

The isolated cells were washed twice with a solution of 0.1% potassium chloride and 0.1% ammonium oxalate. After washing they were resuspended in physiological sodium chloride solution and made to a volume adjusted to the minimal amount required in each individual experiment. Usually the cell suspension was kept in the refrigerator overnight. The cells were still alive after 12 hr., as could be demonstrated by phagocytosis. In a few instances the material was worked up on the day of isolation.

In some of the experiments, the cell suspension was dialyzed in a collodion bag against distilled water for 4 hr. at refrigerator temperature. The water was changed every half hour during the first 2 hr., and hourly for the rest of the period. The cell suspension was kept in the refrigerator overnight, after the dialysis was discontinued. The dialysis was not complete after 4 hr., since there were always still traces of inorganic phosphorus determinable. However, it seemed to be advisable not to extend the dialysis beyond the time indicated above, because of the possibility of weakening the enzymatic activity of the cell suspen-

sion. Cori *et al.* (15) made the observation that liver extracts lose some of their potency after dialysis. Even the procedure of washing the cells twice with potassium chloride and ammonium oxalate seemed to change the quantitative conditions of the experiments.<sup>4</sup> We preferred to work on washed cell material, in order to be sure that the remainder of the diastase-containing plasma was removed. It could be shown in a special experiment, that even after three washings there was still a plasma concentration of about 1:300 detectable by nitrogen determinations. However, the dilution of the plasma in the experiments on horse blood amounted to about 1:10,000 after two washings. Furthermore, horse blood contains so little diastase that the small remainder of highly diluted plasma between the cells is practically free of diastase. The supernate after the second washing never revealed any diastatic activity.

### *Enzymatic Technique*

The number of white blood cells was counted before the reaction mixture was prepared for incubation. In spite of varying differences of the cell count before and after dialysis, the results on dialyzed cell suspensions are based on the count after dialysis. The number of white blood cells varied in the different experiments within a range from half a billion to about seven billions. For the quantitative evaluation of the experiments, counting the white blood cells in a suspension of known volume is superior to the determination of the dry weight of the white blood cell layer. In the cell suspension there are present other cellular admixtures which vary from experiment to experiment.

In the reaction mixture the volume of the cell suspension amounted to two-thirds (occasionally three-fourths) of the total. The other third or fourth consisted of varying additions which are indicated in each individual experiment. The incubation was carried out in a water bath of

<sup>4</sup> Not only the basic glycogen value of unwashed cells is much higher (in one experiment 112 mg./10 billion cells), but also the absolute figures of glycogen degradation during subsequent incubation of the dialyzed material exceed the values on washed cells by a considerable amount. It could also be shown that the lactic acid formed during incubation of unwashed cells is almost twice as high as that on washed cells. In another experiment the two wash fluids were analyzed for lactic acid and reducing substances. There was a considerable amount of lactic acid present (about 12 mg./10 billion cells), but no reducing substances. Glycogen obviously breaks down to lactic acid during the washing procedure at room temperature. Intermediary products do not accumulate. This is consistent with the results on undialyzed cells (cf. later).



37°C. under gentle mechanical shaking, in most instances in an atmosphere of nitrogen. The pH of the reaction mixture ranged between 6.0 and 6.4.

In portions of the reaction mixture, glycogen, inorganic phosphorus, reducing substances, and lactic acid were determined in duplicate before the incubation, and in certain time intervals during the incubation; in some of the experiments all four were determined, in some only one or the other. The glycogen determination was made according to the same method as in our previous studies (4). The cell suspension was heated with sodium hydroxide instead of potassium hydroxide, for reasons which are discussed by Cori, Cori, and Colowick (15). The reduction was determined according to the method of Somogyi (17), the inorganic phosphorus according to Fiske and Subbarow (18), reducing substances according to the Somogyi method (17), and lactic acid according to Barker and Summerson (19). All the results are calculated for 10 billion white blood cells.

### EXPERIMENTAL RESULTS

The basic values of glycogen vary from experiment to experiment. Only the decrease of glycogen and the formation of lactic acid are recorded in the tables of this report. Most of the experiments were carried out on white blood cells of the horse, some on white blood cells isolated from dog blood, and some on cells isolated from human blood. The basic average glycogen value of horse blood is 32.7 mg., that for dog blood 31.4 mg., and that for human blood 24.8 mg./10 billion white blood cells. Out of 32 glycogen determinations on horse blood leucocytes there were 22 with values between 10 and 30 mg./10 billion cells; in one instance the value was between 5 and 10 mg., in one instance between 35 and 40 mg., and in seven samples between 70 and 85 mg. In unwashed cells a maximal value of 112 mg./10 billion cells was reached. Occasionally the observation was made that the basic glycogen values in phosphate buffer were considerably lower than those in saline before the beginning of the incubation; in one experiment, for instance, 13.3 mg./10 billion white blood cells in phosphate, and 25.8 mg. in saline. There might be an immediate breakdown in the phosphate specimens at room temperature.

Specimens of the cell suspension taken immediately after the dialysis, before the reaction mixtures were prepared, showed higher yields of glycogen than the basic values of the reaction mixtures, for instance 15 mg. compared with 12.1 mg. in one experiment, and 29.8 mg. compared with 25.8 mg. in another. A small loss of glycogen can be noticed by keeping the cell suspension in saline in the refrigerator overnight (29.8 mg. immediately after dialysis and 28.0 mg. after keeping overnight at 5°C.).

### 1. Glycogen Degradation in Suspensions of White Blood Cells

The first group of experiments deals with the influence of phosphate ions on the glycogen degradation in suspensions of white blood cells in physiological sodium chloride solution. Adenosine-5-phosphoric acid was added to the specimens containing phosphate. In the control experiments the cell suspension was made to the same volume by addition of normal saline solution (cf. Table I).

TABLE I

*Influence of Phosphate Ions on the Glycogen Degradation in White Blood Cells*

0.001 M Adenosine-5-phosphoric acid added to the specimens containing phosphate. Range of pH in the reaction mixture from 6 to 6.4. Results calculated per 10 billion leucocytes.

Expt. no.	Species	Incubation time	Glycogen disappeared in		Increase of glycogen degradation in phosphate	Lactic acid formed in		Increase of lactic acid formation in phosphate
			Phosph.	Saline		Phosph.	Saline	
		min.	mg.	mg.	%	mg.	mg.	%
251	Dog	120	42.7	31.5	35.6	—	—	—
252	Dog	135	23.5	16.6	41.5	—	—	—
253	Human	15	0.0	0.0	—	—	—	—
		30	4.7	1.1	327.0	—	—	—
		60	6.4	6.4	0.0	—	—	—
		120	11.8	8.4	40.5	—	—	—
254	Dog	30	4.4	4.7	0.0	—	—	—
		60	9.0	6.3	42.8	—	—	—
		120	15.4	13.3	15.8	—	—	—
		150	16.3	15.2	7.2	—	—	—
274	Horse	30	17.4	13.8	26.0	10.4	4.7	121.0
		90	28.1	17.7	58.8	18.3	9.9	84.9
		15 hr.	53.6	32.0	67.5	46.2	29.6	56.2

In Expts. 251 through 254: heparinized, in Expt. 274: citrated blood. Leucocytes isolated in Expts. 251 and 252 in Cushman tubes, in Expts. 253 and 254 by the flotation method and in Expt. 274 by centrifuging the plasma. Phosphate concentration in Expts. 251, 253, and 254, 0.05 M, in Expts. 252 and 274, 0.005 M. Incubation at 37°C. in Expts. 251 through 254, in air; in Expt. 274, in nitrogen.

### 2. Glycogen Degradation in White Blood Cells Dialyzed Against Distilled Water

This series of experiments was done under the same experimental conditions as the previous experiments on undialyzed cell suspensions.

Dialysis was introduced in order to remove phosphate ions. In addition to adenosine-5-phosphoric acid, magnesium sulfate was added to the specimens containing phosphate.

In Table II the changes of the glycogen content of the leucocytes are recorded. The figures represent the results of seven experiments on white blood cells of the horse. The leucocytes were isolated by allowing 2500 ml. citrated blood (final concentration of sodium citrate 0.5%) to settle at room or refrigerator temperature. Duplicate analyses of glycogen were carried out at intervals of 30, 60, 90, and 120 min. In several instances the supernatant liquid of the second washing was tested for diastase against a glycogen solution of known concentration as substrate. No diastatic activity could be demonstrated.

TABLE II

*Influence of Phosphate Ions on the Glycogen Degradation in Washed White Blood Cells Dialyzed Against Distilled Water*

Cells isolated from 2500 ml. citrated horse blood. Volume of cell suspension  $\frac{2}{3}$  of total volume. 0.001 *M* adenosine-5-phosphoric acid, and 0.01 *M* magnesium sulfate added to the specimens containing phosphate. Control experiments in physiological sodium chloride solution. Range of pH in the reaction mixture from 6.0 to 6.4. Incubation in nitrogen at 37°C. Results calculated per 10 billion leucocytes.

Expt no	Incubation time	Glycogen disappeared in			Increase of glycogen degradation in phosphate
		Phosphate		Saline	
		0.005 <i>M</i>	0.05 <i>M</i>		
	<i>min.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg</i>	<i>%</i>
267	120	—	12.3	2.9	324.0
268	120	—	8.4	4.7	78.7
272	60	7.7	-	5.7	35.0
	120	14.4	—	—	—
275	90	7.7	-	2.6	196.1
277	30	8.3	-	0.0	
	90	12.3		2.0	515.0
277 <sub>1</sub>	30	11.3	-	5.7	98.3
281	30	—	15.3	4.4	248.0
	90	—	24.9	12.9	92.9
	120	—	24.9	13.5	84.3

While the concentration of adenosine-5-phosphoric acid was kept at 0.001 *M* and that of magnesium sulfate at 0.01 *M* throughout all experiments, the phosphate concentration was either 0.005 *M* or 0.05 *M*. By lowering the phosphate buffer concentration from 0.05 *M* to 0.005

*M* we tried to improve the experimental conditions for demonstrating a decrease of inorganic phosphorus without impairing the glycogen breakdown. The influence of the phosphate buffer concentration on the quantity of degraded glycogen is shown in Table III.

Similar results were obtained on dialyzed white blood cells of the dog. In Expt. 280, carried out under the same experimental conditions as the experiments on horse blood, the decrease of glycogen amounted to 7.7 mg./10 billion white blood cells after 30 min. incubation in physiological saline and to 17.8 mg. in 0.05 *M* phosphate (+131%), and after 90 min. incubation to 17.6 mg. in saline and to 25.4 mg. in 0.05 *M* phosphate (+44.2%).

If adenosine-5-phosphoric acid was omitted in the reaction mixture (Expt. 303), the glycogen breakdown was far below that occurring in

TABLE III  
*Influence of the Phosphate Concentration on the Glycogen Degradation  
in Dialyzed Suspensions of Leucocytes of Horse Blood*  
Results in mg./10 billion cells

Time	0.005 <i>M</i> phosphate	0.05 <i>M</i> phosphate	Increase of glycogen de- gradation in 0.05 <i>M</i> phos- phate
	mg	mg	%
After 30 min.	10.4 (av. from 4 detns.)	17.8 (av. from 3 detns.)	71.2
After 90 min.	13.3 (av. from 5 detns.)	22.3 (av. from 6 detns.)	67.6
After 120 min.	10.6 (av. from 4 detns.)	15.3 (av. from 9 detns.)	44.3

the presence of this coenzyme. We found after 30 min. incubation 9.40 mg. glycogen disappearance/10 billion white blood cells, and 12.09 mg. after 90 min. incubation in the presence of adenosine-5-phosphoric acid. In the specimens without adenylic acid we found only 3.34 mg. and 6.79 mg., respectively.

*In none of the experiments was a decrease of inorganic phosphorus demonstrable during the incubation of the undialyzed or dialyzed white blood cells suspensions (cf. Part II of this series).*

## DISCUSSION

The experimental data permit the conclusion that the glycogen breakdown in white blood cells follows a pattern similar to that in liver

or muscle. *It is not the result of diastatic activity, but rather of the presence of a phosphorylase. The degradation of glycogen is strongly enhanced in the phosphate-containing specimens of undialyzed white blood cells, most striking in the experiment on white blood cells of the horse (Expt. 274)* Even after 15 hr. incubation the glycogen degradation in saline is still smaller in quantity than that in phosphate buffer. The difference is less pronounced in the experiments on white blood cells of the dog and the human. Particularly in Expt. 254, the rate of glycogen disappearance in saline gradually increases during the time of incubation. It must be emphasized that there is always inorganic phosphorus present in undialyzed cell material. Even dialysis does not result in the complete removal of inorganic phosphorus.

It was shown that this breakdown is not the result of diastatic activity of the small amount of plasma remaining after two washings of the cell suspension. This possibility is excluded, not only by the absence of a reducing sugar and the presence of lactic acid, but also by the absence of diastatic activity in the second wash fluid. Even undiluted horse plasma contains relatively small amounts of diastase as compared with dog or human plasma. Dog plasma contains the strongest concentration of diastase of the three.

In the experiment on horse blood, glycogen and lactic acid<sup>5</sup> were determined at the same intervals. The differences in lactic acid between the phosphate and saline specimens correspond to those of the glycogen figures. There is a marked enhancement of the lactic acid formation in the suspension to which phosphate has been added. *In no instance could a reducing substance be detected in the reaction mixture of undialyzed cell suspensions during incubation.* The increase of lactic acid during the incubation was somewhat smaller than the amount of glycogen which disappeared.

*The glycogen degradation in dialyzed suspensions of white blood cells in phosphate exceeds that in physiological salt solution by a considerable amount as it does in undialyzed material. There is from 35 to 515% more glycogen breakdown in the presence of phosphate ions than in saline.*

The maximal disappearance of glycogen in 10 billion washed and dialyzed white blood cells of the horse, during incubation in nitrogen at 37°C., amounted to about 25 mg. within 90 min. Only in unwashed cell material were larger decreases observed: 37.7 mg. within 30 min.

<sup>5</sup> The basic lactic acid value before the beginning of the incubation was 9.03 mg./10 billion white blood cells.

and 41.7 mg. within 90 min. However, based on the initial value of 112 mg. this is only about 35%, while on washed cells the breakdown reaches occasionally 80–100% of the glycogen values before incubation.

Even after 15 min. of incubation a considerable breakdown of glycogen was noticeable. The maximum seems to be reached between 90 and 120 min. By extending the incubation time up to 15 hr., there was still about one-third of the glycogen left. Addition of 0.2 *M* sodium fluoride did not change the breakdown of glycogen, but inhibited the formation of lactic acid (cf. Part II of this series).

As far as the influence of the pH on the reaction is concerned, the experiments indicate that active glycogen degradation occurs within a range of the pH values of the reaction mixture from 6.0 to 6.4.

The figures in Table III demonstrate that *more glycogen breaks down if more phosphate enters into the reaction*. As will be shown in Part II of this report, these data are well corroborated by quantitative differences in the formation of a reducing substance during the incubation in 0.005 *M* and 0.05 *M* phosphate, respectively.

The diminished glycogen disappearance in the absence of adenosine-5-phosphoric acid supports the concept that the glycogen degradation in white blood cells is catalyzed by a phosphorylase.

### SUMMARY

1. The breakdown of glycogen in polymorphonuclear leucocytes is not the result of diastatic enzyme activity. It is catalyzed by a phosphorylase.

2. With increasing concentration of phosphate ions in the incubation mixture the rate of glycogen degradation increases.

3. In the intact (undialyzed) white blood cell no reducing intermediary products between glycogen and lactic acid accumulate.

4. No decrease in inorganic phosphorus was demonstrable during the incubation of leucocyte suspensions.

5. Adenosine-5-phosphoric acid constitutes an essential activator of the phosphorylase in the reaction between glycogen and phosphate ions in white blood cells.

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# Effects of Glutamic Acid, Lysine and Certain Inorganic Ions on Bovine Alkaline Phosphatases

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## INTRODUCTION

Anions (borate, phosphate, arsenate) inhibit the alkaline phosphatase from bovine intestinal mucosa more than the phosphatase from milk, whereas the reverse is true of the cation ethanolamine (1). Inhibition of both enzymes by anions is competitive with the substrate; that of cations is noncompetitive (1). Glutamic acid inhibits the alkaline phosphatase from rat intestine more than the phosphatases from bone and kidney; the reverse is true of the basic amino acids lysine and histidine (2).

It has been suggested (1) that the relative effects of anions and cations might distinguish two types of alkaline phosphatase. With this in mind, the effects of glutamic acid and lysine, as well as carbonate and ammonium ions, on the bovine phosphatases have been investigated. The type of inhibition exerted by the amino acids has also been determined.

## EXPERIMENTAL METHODS

### *Preparation of the Phosphatases*

Preparation of the milk enzyme has been described (1). The stock solution contained 0.14% protein, and when assayed in a dilution of 1:25 gave a reading of 12.0.

The stock solution of the intestinal mucosa enzyme (1, 3) contained 10 mg./100 ml., and when assayed in a dilution of 1:25 gave a reading of 32.0.

### *Assay of Alkaline Phosphatase (1)*

The assay was performed in 5 min. at 37° in a volume of 12.0 ml., with 0.0025 *M* magnesium chloride and with 0.00075 *M* phenyl phosphate as the substrate. The

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phenol released was determined colorimetrically. The results are reported as photometer readings (logarithmic scale) or as moles of phenol.

To lessen interference on the part of the buffers, the experiments with glutamic acid were performed in ethanolamine at pH 9.6, the optimum for the mucosa enzyme, and at pH 9.9, the optimum for the milk enzyme (1), and the experiments with lysine were performed in carbonate. The optimum pH for the milk enzyme in the carbonate buffer used is 9.5. An optimum of 9.6 in 0.21 *M* carbonate buffer has been reported (4). The mucosa enzyme in the carbonate buffer has an optimum pH of 9.3.

## RESULTS

### Glutamic Acid

Figure 1 shows the inhibitory effect of glutamic acid on the mucosa phosphatase at pH 9.67. The reciprocals of the photometer readings

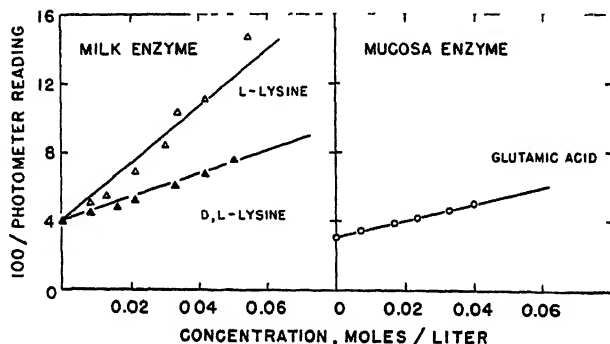


FIG. 1. Effect of glutamic acid on mucosa phosphatase (1:25) in 0.068 *M* ethanolamine, pH 9.67, and effect of lysine on milk phosphatase (2:25) in 0.068 *M* carbonate, pH 9.51.

are plotted against concentration of inhibitor. By extrapolation, a concentration of 0.070 *M* would give 50% inhibition. Glutamic acid was not inhibitory to the milk enzyme at either pH 9.6 or 9.9. A concentration of 0.040 *M* gave a 17.0% average increase in activity, but a concentration of 0.060 *M* produced only 15.0% increase.

Figure 2 shows the effect of 0.032 *M* glutamic acid on the mucosa enzyme with several concentrations of substrate. Comparison of these data with the results of similar experiments without glutamic acid shows that the inhibition was both competitive and noncompetitive (1). Without glutamic acid, the enzyme-substrate constant  $K_s$  was 0.00015 *M*; with glutamic acid, the apparent  $K_s$  was 0.00019 *M*. The

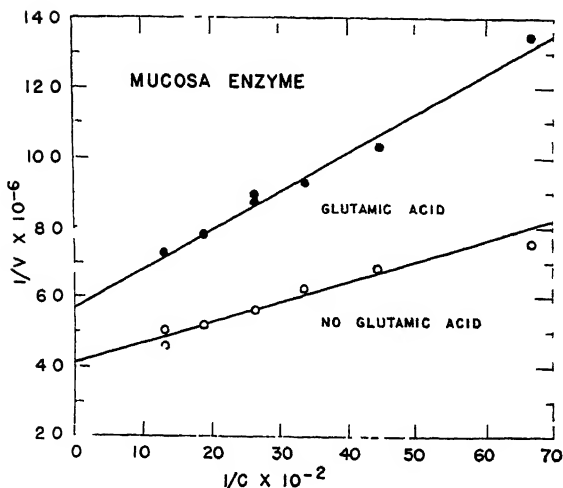


FIG. 2. Effect of glutamic acid on mucosa phosphatase (1:25) in 0.071 *M* ethanolamine, pH 9.67, in relation to the concentration (*C*) of the substrate phenyl phosphate.

maximum velocity ( $V_{\max}$ , from  $1/V$  intercept) decreased from 2.47 to  $1.76 \times 10^{-7}$  mol./5 min.

### *Lysine*

Figure 1 shows the effect of lysine on the milk phosphatase at pH 9.5. A reagent and inactive-enzyme blank were included with every assay. Blank values were especially high with lysine, which may explain the less regular data obtained with lysine (Figs. 1 and 3). A preparation of DL-lysine was one-half as inhibitory as a preparation of L-lysine, the 50% inhibition concentrations being 0.062 and 0.027 *M*, respectively. Comparison of DL-alanine and L-alanine showed that they were equally inhibitory with both milk and mucosa phosphatases.

Lysine was not inhibitory to mucosa phosphatase at either pH 9.3 or 9.5; 0.021 and 0.042 *M* of L-lysine had a negligible effect.

Figure 3 shows the effect of lysine on milk phosphatase in carbonate with several concentrations of substrate. Experiments are included for the enzyme in carbonate without lysine. The enzyme-substrate constant  $K_s$  in carbonate buffer was 0.00039 *M* and the  $V_{\max}$  was  $3.6 \times 10^{-7}$  mol./5 min. Analysis of the lysine experiments at *high* concentra-

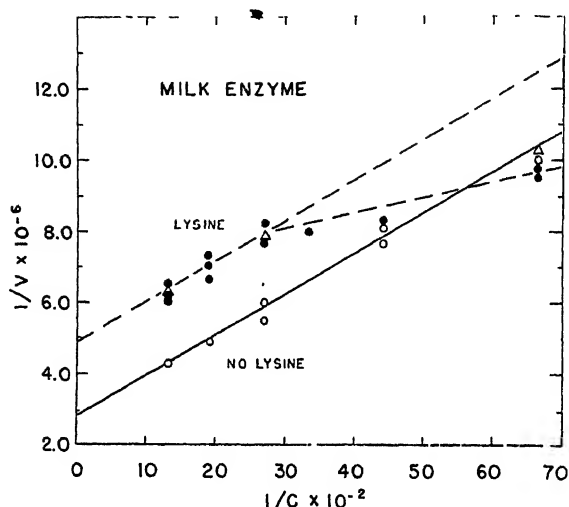


FIG. 3. Effect of lysine on milk phosphatase (2:25) in 0.071 *M* carbonate, pH 9.51, in relation to the concentration (*C*) of the substrate phenyl phosphate. ○, no lysine; ●, 0.033 *M* DL-lysine; △, 0.017 *M* L-lysine.

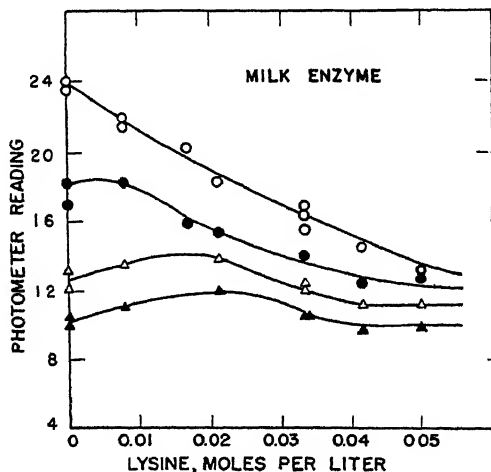


FIG. 4. Effect of DL-lysine on milk phosphatase (2:25) in carbonate, pH 9.51, with different concentrations of the substrate phenyl phosphate. ○, 0.00075 *M* substrate; ●, 0.00038 *M*; △, 0.00023 *M*; ▲, 0.00015 *M*.

tions of substrate led to about the same  $K_s$  value, but the  $V_{\max}$  had decreased to  $2.1 \times 10^{-7}$  mol./5 min. This is characteristic of noncompetitive inhibition. It is not certain, however, that some competitive inhibition was not masked by the stimulation which lysine began to show at low concentrations of substrate. DL- and L-lysine were alike in showing this effect (Fig. 3). Figure 4 shows the results obtained with other concentrations of lysine and several substrate concentrations.

### *Carbonate*

The activity of the mucosa and milk phosphatases was determined in 0.068, 0.034, and 0.017 *M* carbonate-bicarbonate buffers with the pH carefully controlled at 9.51. The respective assay readings in these buffers were: for milk enzyme (2:25) 23.7, 25.2, and 26.6; for mucosa enzyme (1:25) 15.5, 19.0, and 20.0. The data obtained were plotted in the manner shown in Fig. 1. The concentration causing 50% inhibition of the milk enzyme was 0.47 *M*; the concentration producing the same inhibition of the mucosa enzyme was 0.15 *M*. In earlier experiments, in which carbonate was added to the mucosa enzyme in ethanolamine buffer, a value of 0.21 *M* was obtained (1).

### *Ammonium Ion*

Ammonium hydroxide-ammonium chloride buffer of 0.068, 0.034, and 0.017 *M* final concentrations was used. Assays were performed at three pH values. The experiments with the mucosa enzyme were in the range pH 9.3–5. The optimum was above pH 9.5. All values fell on the same curve; hence it can be concluded that the ammonium ion exerts little inhibition on this enzyme.

Experiments with the milk enzyme (2:25) were performed in about the same pH range, and here too the optimum was above pH 9.5. Curves through the assay values were parallel, and at pH 9.5 the readings for the different buffer concentrations were 20.6, 22.8, and 23.8, respectively. The value giving 50% inhibition was calculated to be 0.34 *M*.

### DISCUSSION

Table 1 shows the inhibition patterns of the bovine alkaline phosphatases revealed by the present studies, together with data obtained

by Bodansky (2) and Aebi (10, 11) with other phosphatases. The concentration of glutamic acid (0.070 *M*) giving 50% inhibition of the bovine intestinal mucosa phosphatase was higher than that found by Bodansky (2) (0.030 *M*) for rat intestinal phosphatase with glycerophosphate as the substrate. There may be a species difference in the two enzymes, but the difference in the inhibition was in the direction expected for a high (glycerophosphate) and a low (phenyl phosphate) enzyme-substrate dissociation constant (*K*).

TABLE I  
*Inhibition Pattern of Alkaline Phosphatases*

Enzyme	L-Glutamic acid		L-Lysine		Carbonate <sup>a</sup>		Ammonium ion <sup>b</sup>	
	50% inhibition conc.	Type of inhibition	50% inhibition conc.	Type of inhibition	50% inhibition conc.	Type of inhibition	50% inhibition conc.	Type of inhibition
Bovine intestinal mucosa	0.070 <i>M</i>	Largely noncompet.	No effect <sup>c</sup>	—	0.15 <i>M</i>	—	No effect	—
Bovine milk	Stimulation	—	0.027 <i>M</i>	Non-compet.	0.47 <i>M</i>	—	0.31 <i>M</i>	—
Rat intestine (2)	0.030 <i>M</i>	Non-compet.	0.06–0.14 <i>M</i>	—	—	—	—	—
Rat kidney and bone (2)	0.1 <i>M</i>	—	0.01 <i>M</i>	—	—	—	—	—
Rat and horse kidney (10, 11)	0.6 <i>M</i> <sup>d</sup>	—	0.018 <i>M</i> <sup>d</sup>	—	0.25 <i>M</i> <sup>e</sup> 0.22 <i>M</i> <sup>d</sup>	Compet.	0.15, 0.17 <i>M</i> <sup>d</sup>	Non-compet.

<sup>a</sup> Tetraborate, studied previously (1), is more inhibitory than carbonate, but shows about the same relative inhibition for the two bovine phosphatases. The inhibition with both enzymes is competitive.

<sup>b</sup> Ethanolamine is more inhibitory than the ammonium ion, but the inhibition pattern is the same (1).

<sup>c</sup> The term "no effect" is relative, but the maximum concentrations of the inhibitors studied did not exceed 0.1 *M*.

<sup>d</sup> Calculated from data in Ref. (11).

<sup>e</sup> Calculated from data for the 0.015 *M* concentration of the substrate glycerophosphate in Ref. (10).

The relative proportion of the two types of inhibition exerted by glutamic acid was calculated in the manner employed by Bodansky for the effect of glycine on intestinal phosphatase (5). Inhibition by glutamic acid is 100% noncompetitive in an infinitely concentrated substrate solution, and 96% noncompetitive in 0.0020 *M*, decreasing to 74% in 0.00014 *M* phenyl phosphate. The competitive inhibition did not exceed the effect of glycine on rat intestinal phosphatase (5) and glycine lacks an extra free-carboxyl-group. Bodansky, who studied the

effect of glutamic acid on rat intestinal phosphatase with high concentrations of glycerophosphate (0.0127 to 0.0254  $M$ ), observed only noncompetitive inhibition (2). Thus although glutamic acid resembles inorganic anions in being more inhibitory to intestinal mucosa than to milk phosphatase, it is impossible to say whether it is inhibitory because it is an anion, for the inhibition pattern resembles that shown by glycine (5). Inorganic ions (borate, phosphate, and carbonate), on the other hand, are almost exclusively competitive inhibitors (1).

Bodansky (2) found that glutamic acid was inhibitory to rat bone and kidney phosphatases (50% inhibition at about 0.10  $M$ ), the group to which milk phosphatase may belong. A close comparison of the stimulation shown by the milk phosphatase cannot be made because of species and substrate differences. Bodansky (5) employed 0.006  $M$  glycine in his assays for maximum activity; however, the time of hydrolysis exceeded 5 min., and activation was not observed in experiments of 5-min. duration (6). Glycine and alanine gave no activation in the system used in the present studies.

Bodansky (2) found that both lysine and histidine were more inhibitory to rat intestinal phosphatase than to bone and kidney phosphatases. Histidine was much more inhibitory (concentrations giving 50% inhibition were 0.003–0.006  $M$ ) than other amino acids, and its strong affinity for metals might put it in a special category; hence the present studies were limited to lysine as representative of a simpler type of cation.

The apparent optical specificity shown by lysine in its inhibition of milk phosphatase has not been reported for inhibition of phosphatase by other amino acids. Only *L*- $\alpha$ -amino acids are inhibitory to arginase (7), which is understandable for the competitive lysine and ornithine, but it is less understandable for amino acids that are noncompetitive inhibitors.

Lysine is more inhibitory to mucosa than to milk phosphatase, as are the cations ethanolamine (1) and ammonium ion. The noncompetitive inhibition in concentrated solutions of the substrate is the same type as that shown by cations and also the type shown by lysine to the rat phosphatases (2). This is also the type of inhibition shown by other amino acids (2, 5). The shift from inhibition to stimulation of the milk phosphatase by lysine at low concentrations of substrate has not been observed for any other inhibitors of alkaline phosphatase. The change

in the effect of lysine may be related to the fact that the optimum pH of alkaline phosphatase shifts to lower values with decreasing concentrations of substrate (8) and accordingly experiments at low substrate concentrations were above the pH optimum. Support for this explanation was obtained by experiments above the pH optimum for the highest concentrations of substrate (0.00075 *M*) employed in the experiments presented in Fig. 4. At pH 9.66, the effect of lysine was that shown by the curve for 0.00038 *M*, that is, low concentrations of lysine stimulated the enzyme. At pH 9.35, the lysine had the same effect as at the pH optimum (pH 9.51), that is, inhibition at all concentrations of lysine.

The carbonate anion was more inhibitory to the mucosa than to the milk phosphatase, but the reverse was true for the ammonium cation, which followed the pattern previously observed for inorganic ions (1). Carbonate and ammonium buffers were about equally inhibitory to milk phosphatase and to kidney phosphatase, but the mucosa phosphatase was more inhibited by the carbonate buffer. Some reports (9) have given the impression that ammonium ion is more inhibitory than carbonate ion. Carbonate is more inhibitory to the kidney phosphatase (11) than it is to the milk phosphatase, but this would be expected of a competitive inhibitor with substrates of the different *K<sub>s</sub>* values shown by glycerol- and phenyl phosphates. However, the noncompetitive ammonium ion showed about the same relative difference.

#### SUMMARY

Glutamic acid was more inhibitory to the alkaline phosphatase of bovine intestinal mucosa alkaline than to milk phosphatase; the reverse was true of lysine. Glutamic acid inhibition was predominantly non-competitive; the inhibition pattern resembled that of glycine rather than the competitive inhibition shown by inorganic anions. Lysine activated milk phosphatase at low substrate concentrations.

Carbonate ion was more inhibitory to mucosa phosphatase than to milk phosphatase; the reverse was true of inhibition by the ammonium ion.

The over-all inhibition pattern suggests that there are two types of alkaline phosphatases. One is the intestinal enzyme; the milk enzyme, which is similar to the bone and kidney phosphatases, is the other.

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## The Effect of Streptomycin on a Variant of *Torula utilis*

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### INTRODUCTION

Yeasts in general have been reported to be resistant to the action of streptomycin; only with extremely dilute inocula and high concentrations of streptomycin has any inhibition of growth been observed (1). A variant of *Torula utilis* which utilizes inositol as readily as glucose was isolated in this laboratory for the purpose of studying inositol metabolism. This organism, which will be referred to as *Torula utilis* I-M, in contrast to its parent strain and to other yeasts, is almost completely inhibited at a level of 13 units of streptomycin/ml. The sensitivity of *T. utilis* I-M to streptomycin raised several interesting questions. It seemed possible that the observed inhibition might be due to interference with the conversion of inositol to glucose or a glucose-intermediate. Accordingly, several such compounds were tested for possible reversal of the inhibition. Of particular interest are the observations that D-glucose and D-fructose have no antistreptomycin effect while D-glucose-1-phosphate and D-fructose-1,6-diphosphate, do interfere with the inhibition of *T. utilis* I-M by streptomycin.

In an extension of these studies to bacteria it was discovered that both D-glucose-1-phosphate and D-fructose-1,6-diphosphate effectively reverse the inhibition of *Lactobacillus arabinosus* and *Staphylococcus aureus* by streptomycin. No previous workers have reported studies on the relationship of the hexose phosphates to streptomycin activity, either with bacteria or yeasts. These results therefore open up a new approach to the problem of the mechanism of action of streptomycin.

## EXPERIMENTAL AND RESULTS

*Isolation of Torula utilis I-M*

The basal medium employed had the following composition:

Substance	Amount/500 ml. of double strength medium
Casamino acids	5.0 g.
Inositol	10.0 g.
$K_2HPO_4 \cdot 3H_2O$	1.14 g.
$KH_2PO_4$	1.36 g.
$(NH_4)_2SO_4$	1.0 g.
$CaCl_2 \cdot 6H_2O$	0.125 g.
$MgSO_4$	0.125 g.
$FeCl_3$	2.5 mg.
$MnSO_4$	2.5 mg.
Thiamine HCl	0.25 mg.
Biotin	0.001 mg.
Calcium pantothenate	0.5 mg.
Pyridoxine HCl	0.1 mg.
Nicotinic acid	0.2 mg.
Final pH of medium	6.5

The culture was maintained on agar slants composed of 0.5% peptone, 0.3% yeast extract, 1.0% inositol, 0.1%  $KH_2PO_4$ , and 1.5% agar.

A culture of *T. utilis* obtained from the Horticulture Department of the University of Illinois was used to inoculate 10 ml. of basal medium in a 125-ml. Erlenmeyer flask, which was incubated at 30°C. for 4-5 days, when growth was perceptible. The suspension was streaked on agar plates which were incubated at 30°C. until colonies appeared. These were transferred to liquid medium. This procedure was repeated over a period of 3 months. Then transfers were made in the basal medium every 24 hr. for 3-4 weeks. A rapidly growing variant utilizing 90-5% of the medium inositol in 64-72 hr. was thus isolated. The variant did not lose its ability to utilize inositol and retained the same degree of sensitivity toward streptomycin after six subcultures in 1% glucose medium.

Mutants of a culture of *Hansenula anomala* and another strain of *T. utilis* which grow on inositol in place of glucose have been isolated by this same procedure. These have also been found to be sensitive to streptomycin.

*Inhibition by Streptomycin*

The inoculum was standardized as follows. One loopful of cells from a slant was used to seed 10 ml. of basal medium in a 125-ml. Erlenmeyer flask, which was incubated at 30°C. for 24 hr. Sterile water (15 ml.) was added, and 1 ml. of this suspension was used per flask. The optical density of the inoculated medium before incubation ranged from 0.08-0.11.

TABLE I

1 Comparison of the Sensitivity of *Torula utilis* and *Torula utilis* I-M toward Streptomycin in 1% Glucose Medium

Streptomycin units/ml	Optical density	
	<i>Torula utilis</i>	<i>Torula utilis</i> I-M
0	0.53	0.92
1.3	0.49	0.85
3.3	0.48	0.63
6.6	0.50	0.25
13.2	0.51	0.15
33.3	0.49	0.13
66.6	0.48	0.14

The inositol in the basal medium was replaced by different carbohydrates, as indicated in Table II. Ten ml. of medium was autoclaved in 125-ml. Erlenmeyer flasks at 15 p.s.i. for 20 min. One ml. of a streptomycin solution, prepared in sterile water, was added. Each flask was inoculated with 1 ml. of the cell suspension. After 16 hr. of incubation at 30°C., the turbidity was determined in colorimeter tubes using the Evelyn photoelectric colorimeter with the 620 m $\mu$  filter.

TABLE II

Effect of Streptomycin on Growth of *Torula utilis* I-M in Different Media

Streptomycin	Optical density and test medium					
	Inositol 1%	Glucose 1%	Fructose 1%	Galactose 1%	Sucrose 1%	Rabbit liver glycogen 1%
units/ml						
0	1.00	0.92	1.00	1.00	1.02	0.41
1.3	0.74	0.85	0.96	0.98	0.96	0.10
3.3	0.27	0.63	0.43	0.38	0.36	0.10
6.6	0.15	0.25	0.21	0.20	0.20	0.10
13.2	0.11	0.15	0.14	0.11	0.12	0.10
33.3	0.11	0.13	0.12	0.12	0.11	0.10
66.6	0.10	0.12	0.12	0.11	0.12	0.11

#### Reversal of Streptomycin Inhibition

*Torula utilis* I-M. The procedure described for the inhibition studies was applied with a slight modification. Double strength basal medium and solutions of the compounds tested were sterilized separately. Compounds unstable to heat as *meso-scylo*-inosose, K-D-glucose-1-phos-

phate, K-D-fructose-1,6-diphosphate, and sodium adenosinetriphosphoric acid ( $\text{Na}_4\text{ATP}$ ) were dissolved in sterile water. Solutions of the other compounds were steamed for 10 min.

TABLE III

*Effect of Different Compounds on Growth Inhibition of Torula utilis  
I-M by Streptomycin in 1% Inositol Medium*

Solutions of the acids tested were adjusted to pH 7 with potassium hydroxide

Compound	Amount mmole per flask	Optical density			
		Streptomycin (units/ml.)			
		0	6.6	13.2	33.1
None		1.05	0.13	0.10	0.10
D-Glucose	0.2	1.00	0.20	0.15	0.12
D-Fructose	0.2	1.00	0.19	0.16	0.12
D-Galactose	0.2	1.02	0.11	0.10	0.11
D-Mannose	0.2	1.00	0.11	0.11	0.10
L-Sorbose	0.2	0.94	0.11	0.11	0.11
<i>Meso-scyllo</i> -inosonic	0.2	1.02	0.14	0.15	0.14
Na- $\beta$ -glycerophosphate	0.2	0.90	0.12	0.09	0.10
L-Glutamate	0.2	1.15	0.17	0.12	0.09
Succinate	0.2	1.02	0.41	0.23	0.14
Fumarate	0.2	1.05	0.24	0.14	0.10
Malate	0.2	0.92	0.19	0.13	0.10
D-Glucose-1-phosphate	0.2	0.96	0.55	0.24	0.11
D-Fructose-1,6- diphosphate	0.2	1.02	0.89	0.44	0.13
D-Glucose + fumarate	0.2	1.07	0.48	0.21	0.17
	0.2				

### *Lactobacillus arabinosus and Staphylococcus aureus*

The basal medium, broth for the cultures, agar stabs, and slants had the following composition:

Tryptone 1%

Peptone 0.5%

Yeast extract 0.5%

Glucose 1.0%

Salts B 1 ml./100 ml. double strength medium.

Citrate-phosphate buffer 1 ml./100 ml. double strength medium.

Agar 1.5% (for stabs and slants).

Salts B	g./250 ml.
MgSO <sub>4</sub> ·7H <sub>2</sub> O	10.0
NaCl	0.5
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.5
MnSO <sub>4</sub> ·4H <sub>2</sub> O	0.5

Citrate—phosphate buffer.

	g./100 ml
Citric acid	1.9
K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O	11.0
pH adjusted to 7.	

The inocula were taken from 24-hr. broth cultures prepared from agar slabs of *Lactobacillus arabinosus* and slants of *Staphylococcus aureus*. The optical density of these suspensions ranged from 0.52–0.70.

Double strength medium (5 ml.) was pipetted into test tubes and steamed for 10 min. The solution of the compound (5 ml.) was added, followed by 1 ml. of streptomycin solution. The tubes were then seeded with 1 drop of the inoculum. After 18–24 hr. of incubation at 37°C. the turbidity was determined in the Evelyn photoelectric colorimeter with a 620 mμ. filter.

## DISCUSSION

In previous studies of the antibacterial action of streptomycin, glucose, and a number of the Kreb's cycle intermediates have been tested. The results reported by different investigators varied from one test organism to another. With *Escherichia coli*, glucose did not interfere with the bacteriostatic action of streptomycin (2). Fumarate, succinate, and malate supported growth of this organism in the presence of the antibiotic (3). Fumarate also protected *Proteus vulgaris* but not *S. aureus* and *A. aerogenes* (3).

The rate of oxidation of glucose, succinate, malate, fumarate, and oxalacetate by resting cells of *E. coli* was not affected by streptomycin (4). Using this same organism, Umbreit has reported that streptomycin inhibits the condensation of pyruvate or another keto acid with oxalacetate and prevents the formation from fumarate oxidation of a substance that stimulates threonine oxidation (5). Streptomycin had no effect on the aerobic oxidation of glucose and glutamate by resting cells of *S. aureus* but retarded the rate of succinate and fumarate oxidation (6). The oxidation of glucose by resting cells of *B. cereus* (6) and *S. aureus* (7) was inhibited by streptomycin.

In the present investigation glucose did not prevent the growth inhibiting action of streptomycin on *Torula utilis* I-M. Of all the com-

TABLE IV

*Effect of Different Compounds on the Bacteriostatic Action of Streptomycin*  
Solutions of the acids tested were adjusted to pH 7 with potassium hydroxide

Compound		Streptomycin	Optical density	
			<i>L. arabinosus</i>	<i>S. aureus</i>
None		<i>mmole/tube</i>		
		<i>units/tube</i>		
		0	0.24	0.15
		5	0.13	0.05
L-Glutamate	0.2	10	0.11	0.03
		15	0.08	0.02
		0	0.37	0.15
		10	0.35	0.07
Succinate	0.1	0	0.43	0.12
		5	0.40	0.10
		15	0.38	0.08
Fumarate	0.1	0	0.46	0.19
		5	0.37	0.17
		10	0.37	0.05
Malate	0.1	0	0.55	0.21
		5	0.47	0.14
		10	0.42	0.03
Na- $\beta$ -glycerophosphate	0.1	0	0.48	0.30
		5	0.03	0.05
D-Glucose-1-phosphate	0.1	0	0.33	0.17
		5	0.31	0.12
		15	0.25	0.08
D-Fructose-1,6-diphosphate	0.1	0	0.55	0.28
		10	0.51	0.23
		15	0.43	0.22

pounds tested, only the hexose phosphates effectively reversed the action of streptomycin on all three test organisms, namely *T. utilis* I-M, *L. arabinosus*, and *S. aureus*.

Results obtained with the dicarboxylic acids varied with the test organism employed. Succinate was effective in reversing the inhibition

of *T. utilis* I-M by streptomycin, while fumarate was effective only in the presence of glucose. Glutamate, succinate, fumarate, and malate all favored the growth of *L. arabinosus* in the presence of streptomycin. In the case of *S. aureus* only fumarate and malate antagonized the effect of streptomycin.

These results are obviously preliminary in nature. However, they provide the first information regarding the relation of hexose phosphates to streptomycin activity as well as a basis for approaching these questions.

#### ACKNOWLEDGMENTS

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#### SUMMARY

A variant of *Torula utilis* which utilizes inositol has been developed. In contrast to its parent strain, this mutant is sensitive to streptomycin on a glucose or an inositol medium. However the inhibition is reversed by D-glucose-1-phosphate or D-fructose-1,6-diphosphate. The inhibition of *Lactobacillus arabinosus* and *Staphylococcus aureus* by streptomycin is also reversed by either of the hexose phosphates.

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## LETTERS TO THE EDITORS

### Studies on Permeability. II. The Effect of Acetyl Choline and Physostigmine on the Permeability to Potassium of Dog Erythrocytes <sup>1</sup>

In a previous communication (1) we reported that acetyl choline markedly retarded the rate of hemolysis of dog erythrocytes suspended in an isotonic medium containing a high proportion of potassium salts, of pH about 8. When physostigmine was added, the rate of hemolysis was again increased, the degree depending on the concentration of physostigmine.

The present communication describes the prolytic changes which occur in dog erythrocytes when treated with acetyl choline or with acetyl choline plus physostigmine, in an isotonic solution containing mixtures of potassium bicarbonate (0.154 *M*) and potassium chloride (0.154 *M*).

#### METHODS

Freshly drawn dog blood was defibrinated, centrifuged, and the cells washed with the experimental medium. Suspensions of 10% packed cells by volume were used. The final concentration of acetyl choline was 0.01 or 0.005 *M*. The concentrations of physostigmine used are indicated in the figure. The suspensions were incubated at +5°C. and the samples were removed at specified intervals for hematocrit, hemolysis, potassium, sodium, and water determinations. For hematocrit determinations, Bauer-Schenk tubes were used. Hemolysis was measured on the supernatant fluid by means of a photoelectric colorimeter. Potassium and sodium determinations were carried out on a measured volume of packed cells, corrections being made for swelling, by means of a Beckman DU Spectrophotometer with flame attachment. Water determinations were measured as the decrease in weight of packed cells on drying at 110°C.

#### RESULTS

Figure 1 shows curves in which per cent hemolysis of dog cells is plotted against time. At 5°C. the degree of hemolysis is low in all cases

<sup>1</sup> This work was supported by grants from the Mallinckrodt Chemical Works and from the Upjohn Company.



for experiments of short duration, but is significantly higher in the control suspension without acetyl choline and in the suspension with acetyl choline plus the highest concentration of physostigmine than in the others. When the hemolysis was 1% or more, the hematocrit readings were corrected accordingly. It may be seen from Fig. 2, curve 1, that dog erythrocytes swelled rapidly as measured by hemato-

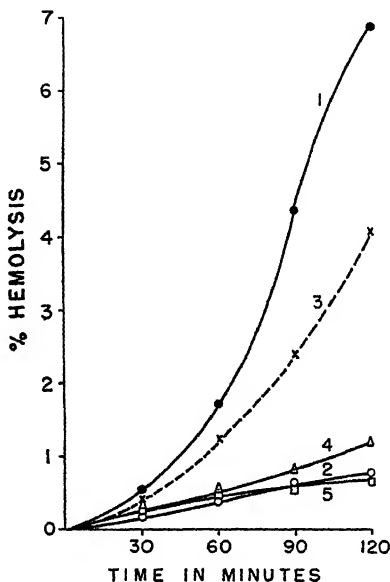


Fig. 1. Dog erythrocytes suspended in a solution consisting of 80 parts by volume isotonic KCl and 42 parts isotonic  $\text{KHCO}_3$ ;  $t = 5^\circ\text{C}$ .

- Curve 1. Control, without acetyl choline ●—●.  
 Curve 2. Control with acetyl choline  $5 \times 10^{-8} M$  ○—○.  
 Curve 3. Physostigmine  $2.7 \times 10^{-6} M$  + acetyl choline ×—×.  
 Curve 4. Physostigmine  $5 \times 10^{-6} M$  + acetyl choline △—△.  
 Curve 5. Physostigmine  $1 \times 10^{-6} M$  + acetyl choline □—□.

crit determinations. The swelling is accompanied by an increase in intracellular water (not graphed) and an increase in intracellular potassium (Fig. 3, curve 1). There was, however, little change in the intracellular sodium in the early stages of the experiment. These results are in agreement with those observed by other workers (2).

When an isotonic solution containing acetyl choline was added to the suspension, there was a marked increase in resistance to swelling and to change in the internal ionic composition of the cell (Figs. 2 and 3, curves 2).

When physostigmine is added to the suspension of erythrocytes it usually causes some slight change in permeability to potassium and

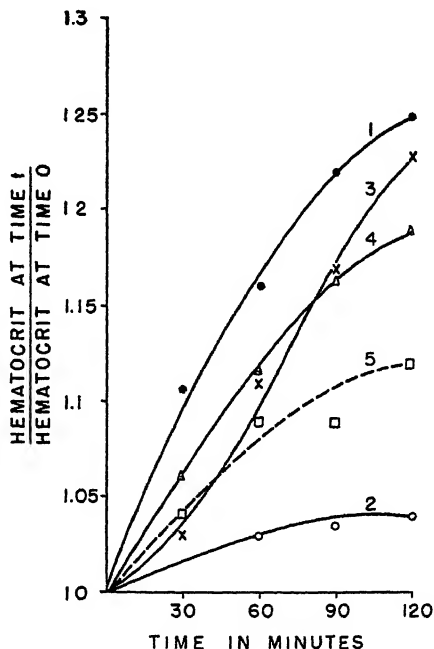


FIG. 2. Dog erythrocytes suspended in a solution consisting of 80 parts by volume isotonic KCl and 42 parts isotonic  $\text{KHCO}_3$ ;  $t = 5^\circ\text{C}$ .

- Curve 1. Control, without acetyl choline ●—●.  
 Curve 2. Control with acetyl choline  $5 \times 10^{-3} M$  ○—○.  
 Curve 3. Physostigmine  $2.7 \times 10^{-6} M$  + acetyl choline ×—×.  
 Curve 4. Physostigmine  $5 \times 10^{-6} M$  + acetyl choline △—△.  
 Curve 5. Physostigmine  $1 \times 10^{-6} M$  + acetyl choline □—□.

water, depending on the composition of the medium and the pH. When added along with acetyl choline, it causes a marked increase in permeability to potassium as well as a concomitant increase in cell volume, when compared with the cells treated with acetyl choline

alone. When the concentration of physostigmine is around  $2.7 \times 10^{-6} M$ , the rate of swelling and of permeability to potassium approaches that seen in the erythrocytes without added substrate (Figs. 2 and 3, curves 3, 4, 5). This concentration inhibits cholinesterase activity by 85%; the second concentration inhibits by 58% at 5°C.

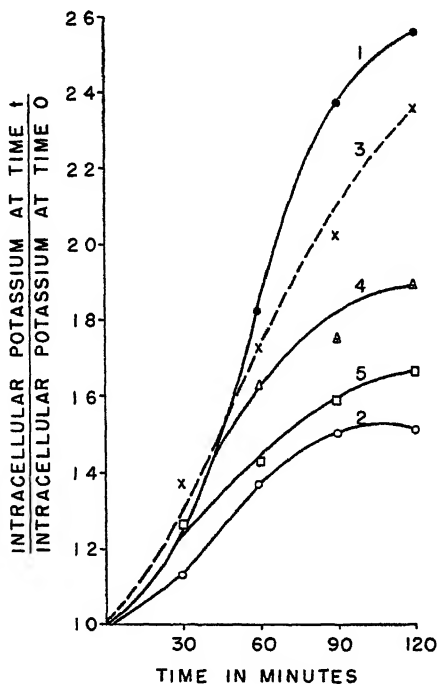


Fig. 3. Dog erythrocytes suspended in a solution consisting of 80 parts by volume isotonic KCl and 42 parts isotonic  $\text{KHCO}_3$ ;  $t = 5^\circ\text{C}$ .

- Curve 1. Control, without acetyl choline ●—●.  
 Curve 2. Control with acetyl choline  $5 \times 10^{-6} M$  ○—○.  
 Curve 3. Physostigmine  $2.7 \times 10^{-6} M$  + acetyl choline ×---×.  
 Curve 4. Physostigmine  $5 \times 10^{-6} M$  + acetyl choline Δ—Δ.  
 Curve 5. Physostigmine  $1 \times 10^{-6} M$  + acetyl choline □—□.

Changes in the sodium content of the cell occur much later in the experiment, usually after 6 hr. In one experiment in a  $\text{KHCO}_3$ -KCl medium, the cell sodium fell to 50% of its initial value. At this point hemolysis was considerable (about 30%), and there was also a decline

in intracellular potassium. The addition of acetyl choline prevents this loss of sodium and the addition of physostigmine allows it to proceed again as in the absence of substrate. Since in the suspensions without acetyl choline or with acetyl choline plus physostigmine, hemolysis is considerable, the apparent fall in intracellular sodium may be due to the remaining resistant cells having a lower sodium content as well as being less permeable to potassium as suggested by Davson (2). Whether the lowered sodium content is a result of a loss of sodium during the experiment or an increased resistance of these cells because of a lower sodium content and a decreased permeability to potassium, is not known.

### SUMMARY

1. The addition of acetyl choline to a suspension of dog erythrocytes in a potassium medium decreases the rate of swelling and the permeability to potassium of the erythrocyte.

2. Physostigmine in concentrations which inhibit cholinesterase activity by 60–80% causes a reversal of the permeability effect produced by acetyl choline.

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## Effect of Foods Grown in Different Areas on the Prevalence of Dental Caries in Hamsters

Fluorine in water supplies is considered to be a principal factor accounting for differences in caries prevalence (1) in human subjects in various parts of the United States. The present study is an attempt to determine whether the development of caries in hamsters is affected by diets containing foods grown in areas where there is a high and low incidence of caries.

A modified Hoppert-Webber-Caniff diet (2) of the following com-

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position was used: corn 63%, dried whole-milk powder 30%, alfalfa 6%, sodium chloride 1%. The corn and milk components of Diet No. 1 were obtained from Orono, Maine, and St. Albans, Vermont, respectively; the soil in both these areas is podzolic. The corn and milk components of Diet No. 2 were obtained from Hereford and Lubbock, Texas, respectively, where the soil is red and yellow. The same alfalfa, from Ithaca, New York, was used in both diets. These diets and distilled water were supplied *ad lib.* to hamsters housed in sheet metal cages resting on wooden flooring. Forty weanling hamsters (19 females, 21 males) were placed on Diet No. 1 while 37 (18 females, 19 males) were placed on Diet No. 2. Each animal was weighed weekly. After 15, 16, 17, 18, 21, and 23 weeks, representative hamsters (a total of

TABLE I

*Incidence and Area of Caries in Hamsters Fed Diets Containing Corn and Whole-Milk Powder from New England or Texas During 15 to 23 Weeks*

	Diet group		
	No. 1 (N.E.)	No. 2 (Texas)	Difference
No. hamsters per group: male	13	14	
female	12	11	
Average number carious teeth	5.8( $\pm 1.67$ ) <sup>a</sup>	3.4( $\pm 1.83$ ) <sup>a</sup>	2.4(41%)
Average number carious surfaces	7.4( $\pm 3.19$ ) <sup>a</sup>	3.9( $\pm 2.65$ ) <sup>a</sup>	3.5(47%)
Caries area (sq. mm. on score chart)	296.7( $\pm 521.7$ ) <sup>a</sup>	113.7( $\pm 205.5$ ) <sup>a</sup>	183. (62%)

<sup>a</sup> Standard deviation.

25 from each group) were autopsied. The heads were fixed in formalin, dried, skinned, and treated with 3% hydrogen peroxide. The teeth were examined, scored and charted for dental caries according to the Keyes procedure (3). The scoring of caries was spot checked by others with past experience with this technique.

The hamsters gained approximately 5 g. weekly on both diets. Caries were noted in the teeth of hamsters from both groups at the first autopsy (15th week). Male hamsters were more susceptible to caries than female hamsters; maxillary teeth were more carious than mandibular teeth; and there was a very striking preponderance of caries in the occlusal pits of the maxillary molars, particularly the second molars. Only two hamsters (both on the Texas diet) were caries-free.

It will be seen in Table I that there was approximately 40% more carious teeth, and approximately 50% more carious surfaces, in the group fed New England corn and milk. Both these differences were studied statistically and found to be significant at the 1% level. It is evident that the area in which foods are grown may affect the prevalence of dental caries. To our knowledge this is the first controlled comparison of the cariogenic effect of the same foodstuffs produced in different soil areas.

Further research will reveal whether the corn, or the milk, or both, were responsible for the differences noted, and investigations are planned to isolate and identify the anti-caries or cariogenic factor.

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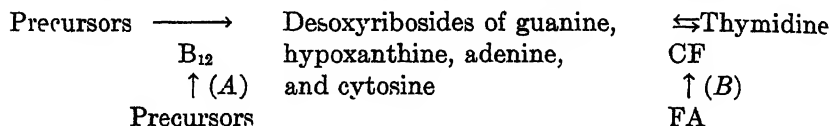
Received December 8, 1949

### Vitamin B<sub>12</sub> and "Citrovorum Factor" in the Nutrition of *Lactobacillus leichmannii* and *Leuconostoc citrovorum*

It has been observed that vitamin B<sub>12</sub> or the desoxyribosides of any of the following: guanine, hypoxanthine, adenine, cytosine, or thymine, will promote growth in *Lactobacillus leichmannii* 313 (1-4) while "citrovorum factor" (CF) or thymidine, but not the other desoxyribosides or B<sub>12</sub>, will permit *Leuconostoc citrovorum* 8081 to grow on a purified culture medium (2,5,6,7). A close relationship between CF and folic acid (FA)\* has been indicated (5,6,7). Recently it has been observed by Bardos and co-workers (8) and independently in this laboratory that CF upon standing in dilute HCl at room temperature is destroyed with the simultaneous liberation of a substance with FA activity. In our experiments (9) a concentrate of CF containing 200,000 units/ml. (5) was found to have a potency for *S. faecalis* corresponding to 20 µg.

\* The term "folic acid" has been substituted for "pteroylglutamic acid" throughout this communication.—Ed.

of FA/ml. When 1 ml. of this solution was added to a solution containing 20  $\mu$ g. of FA, it was possible to separate the CF activity from the added FA by chromatography. Treatment of the original solution of CF with HCl destroyed its activity for *Leuc. citrovorum* but not for *S. faecalis*. Simultaneously the activity of the treated material for *S. faecalis* was no longer separable from FA by chromatography. These findings may indicate that CF is a compound which contains FA. These observations enable the following scheme to be suggested.



On this basis, *L. leichmannii* would be able to carry out step (B) but is unable to carry out step (A). Conversely, *Leuc. citrovorum* may carry out step (A) but not step (B). In support of this, it was found that on purified media *Leuc. citrovorum* produced vitamin B<sub>12</sub> activity while *L. leichmannii* produced CF.

A culture medium (5) was supplemented with alkali-treated liver extract as a source of CF (7). The medium thus supplemented contained 0.03  $\mu$ g. of vitamin B<sub>12</sub> activity/ml. (10). It was inoculated with *Leuc. citrovorum* and after 18 hr. of growth, the culture was neutralized, steamed, and assayed for vitamin B<sub>12</sub>. It was found to contain alkali-labile activity (10) corresponding to 0.12  $\mu$ g. of vitamin B<sub>12</sub>/ml., thus indicating production of vitamin B<sub>12</sub> activity.

The medium used for *L. leichmannii* was that of Hoffmann *et al.* (10) with the asparagus concentrate omitted, with the casein hydrolyzate replaced by an amino acid mixture (11), and containing 20  $\mu$ g. of FA and 10  $\mu$ g. of vitamin B<sub>12</sub>/ml. The medium, which contained less than 0.02 unit (5) of CF activity/ml., was inoculated with *L. leichmannii* 313, incubated for 9 hr., steamed in 0.1 N NaOH for 30 min. and neutralized. The cell extract obtained in this manner was assayed with *Leuc. citrovorum* using a 16-hr. incubation period and was found to contain 2.5 units of CF activity/ml. To an aliquot of the extract, hydrochloric acid was added to pH 2.1 and the solution was allowed to stand at 25° for 2 hr., following which the solution was re-assayed for CF and it was found that only 20% of the original potency remained. This lability to dilute acid is characteristic of CF and served to confirm its presence in the extract.

The ability of thymidine to replace the requirement of *L. leichmannii* for both vitamin B<sub>12</sub> and FA (4) may indicate that the organism is able to synthesize the other desoxyribosides from thymidine as indicated in the above scheme, thus obviating the need of the organism for vitamin B<sub>12</sub> in the production of the other desoxyribosides when thymidine is supplied. Vitamin B<sub>12</sub> formed by *Leuc. citrovorum* may serve in the synthesis of desoxyribosides other than thymidine which in turn act as precursors of thymidine when CF is supplied. With *L. leichmannii*, CF produced by the organism may catalyze the formation of thymidine from the other desoxyribosides which are formed when B<sub>12</sub> is supplied.

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## Enamel Erosive Properties of Fruits and Fruit Juices

A number of investigators (1,2,3) have shown that acid beverages, natural or synthetic, when fed to rats cause destruction of the lingual enamel of the molar teeth.

We find that acid fruits themselves have little or no effect upon the enamel but that the juices made from an equivalent weight of fruit,



or in some cases even half the equivalent, have marked erosive properties.

Healthy young rats fed stock diet and weighing approximately 150 g. were fed 5 different fruits or their juices for 5 days and were killed on the 6th day. Free access to water and food was allowed except for a few rats which were slow in taking the supplements. In these cases water cups or food cups were removed for a part of the time. Five to 10 rats were fed each supplement.

Grapefruit juice and pineapple juice were expressed from sections of the same fruits that were fed in the fresh state. Guava and Java plum juices were prepared by cooking the fruit with water and straining, making literally a watery extract such as is used for jelly making. Green mango "juice" was made by mixing slices of the green fruit with water in a Waring Blendor and squeezing the resulting mixture. All the fruits were fed in the raw uncooked state. Ten to 15% of sugar was added to the fruit and to the juices to make them more acceptable to the rats with the exception of pineapple, pineapple juice, and green mango.

The scores for the molar teeth were determined as recommended by Restarski, Gortner, and McCay (4). The average score for grapefruit was 2.0 but that for the other fruits was 0.6 or less, whereas the scores for the fruit juices ranged from 2.7 for fresh pineapple juice to 4.8 for Java plum juice.

Whether or not the erosive properties are purely a matter of titratable acidity as Muller and Gortner (5) have recently suggested is being studied. A detailed account of this study will be published in the near future.

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## Growth Depression by Ammonium Citrate and Urea, and Its Reversion by Nicotinamide in the Rat

Data indicating ammonia-N retention and probable utilization for protein synthesis, by the rat are well-known. Rose (1) refers to similar results.

The influence of niacin and nicotinamide on the growth of the rat on different protein diets as shown by Krehl *et al.* (2) induced us to try the effect of nicotinamide in low casein-ammonium citrate and low casein-urea rations.

TABLE I  
*Rations Used and Average Weight gain in 44 days*

Group	Ration	Number of animals	Weight gain in 44 days
I	Basal (see text)	6 males	45.6
		2 fem.	49.0
II	Basal+NA	4 males	44.2
		2 fem.	70.0
III	Basal+13% ammonium citrate	5 males	6.6
		4 fem.	9.5
IV	Basal+13% ammonium citrate+NA	6 males	52.5
		5 fem.	48.8
V	Basal+3.4% urea	3 fem.	17.2
VI	Basal+3.4% urea+NA	4 fem.	65.5
VII	18% casein+NA	3 males	138.3
		3 fem.	93.3

### EXPERIMENTAL

Rats, Sprague-Dawley, albino, 21 days, were kept in individual screened-bottom cages.

Basal ration: Labco casein, vitamin-free, 8%; sucrose 83%; peanut oil 5%; salts IV (3) 4%; cystine 0.2%. When supplements were added, the proper amount of sucrose was deducted. Vitamin supplements per 100 g. of ration: B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, PABA, K, 0.5 mg.; Ca pantothenate 2 mg.; folic acid 50 µg., biotin 10 µg.; inositol 10 mg.; choline 100 mg.; A, D, and 2 mg. of  $\alpha$ -tocopherol twice a week by dropper.

Diammonium citrate and urea-supplements were given in amounts equivalent to 10% casein, on N basis. Nicotinamide (NA) supplement 500 mg. mixed with 1 kg. of ration.

The experiments and results are summarized in Table I.

In another experiment, 3 animals on ration III received subcutaneous injection of nicotinamide, 5 mg. daily, for 14 days, from the 10th to the 24th day of experiment, without any growth improvement as compared to controls.

After 44 days, groups were rearranged according to Fig. 1 and observed for 17 days more.

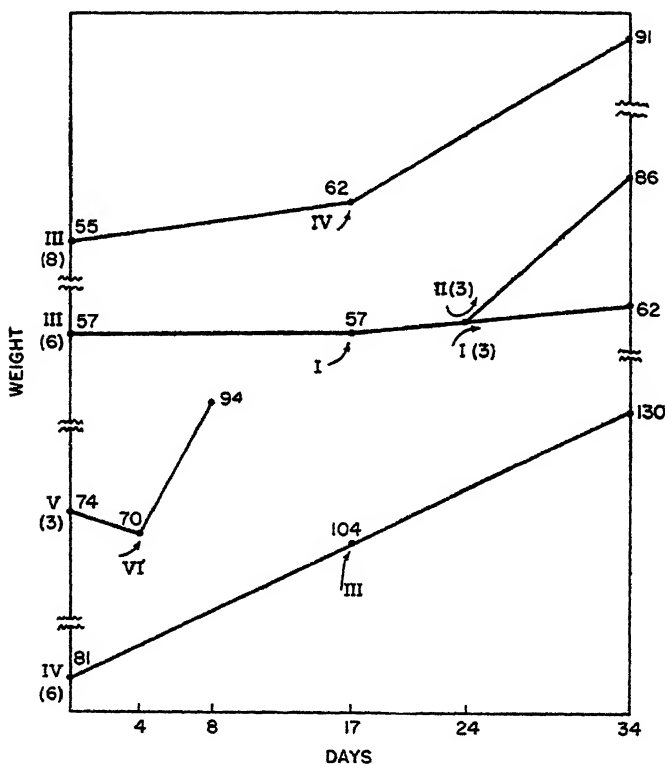


FIG. 1. Growth rates for equal periods before and after changing diets. Roman numerals: diets (according to Table I), Arabic numerals: average weight; numbers in parentheses: number of animals in each group. (The arrows indicate the date of rearrangement of diets.)

Our results show that ammonium citrate or urea, at the tested levels, depress the growth of the young rat on an 8% casein diet. This effect can be reversed by nicotinamide mixed with the ration. Subcutaneous administration was without effect. This suggests that nicotinamide does not act directly, but on the intestinal flora. Further experiments are being conducted.

## ACKNOWLEDGMENTS

We wish to thank The Rockefeller Foundation for Labeo casein, choline, and inositol, Dr. E. Sevringhaus of Hoffmann-La Roche, New Jersey, for biotin. Produtos Roche do Brasil, for  $\alpha$ -tocopherol.

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## Book Reviews

**Biochimie Medicale.** By MICHEL POLONOVSKI, Member of the Academy of Medicine and T. P. BOULANGER, M. MACHEBOEUF, J. ROCHE and C. SANNIÉ, Professors of Biological Chemistry of the Faculty of Medicine, Paris, France. Masson and Co., Paris, France. 4th ed. 1948. 710 pp., 61 figures. Price 1400 francs.

This book has been written with the primary objective of orienting its contents toward human physiology and pathology, with biochemistry being considered as a specialized division of general physiology. However, despite the announced intention of writing this volume principally for students of medicine, the broad scope of its contents recommend it for use by those whose major interest lies in biochemistry.

The volume is divided into 5 sections. The first is concerned with the fundamental constituents of living material, and includes chapters on the organic chemistry of the carbohydrates, the lipides and the proteins. In addition, there is an introductory chapter entitled "Object and Divisions of Biochemistry," in which the composition and properties of living cells are discussed in terms of their morphological characteristics and related to the basic principles of chemistry. In the second chapter, the elementary composition of living matter is presented, while chapter three details the composition with respect to the inorganic substances, *i.e.*, water and salts. The final chapter in this section is entitled "The Biocatalysts," and into these pages has been put the treatment of vitamins, hormones and enzymes.

Section two is headed "The Chemical Phenomena of Digestion," and includes chapters on salivary, gastric and intestinal digestion, as well as a chapter on the formation and composition of feces, including the chemical action of microorganisms.

Section three is entitled "Tissues, Fluids, and Secretions" and is concerned with chapters covering the chemical composition of tissues, blood, milk, tears, sebaceous secretion, sex glands' secretions, and semen.

Section four is concerned with intermediary metabolism, and includes descriptions of methods used for studies in this field, as well as chapters on cellular oxidation, and the metabolism of carbohydrates, lipides and proteins, including a short chapter on the integration of the metabolism of these three classes of substances. The final chapter in this section deals with the metabolism of water and salts.

The fifth section is entitled "Excretion" and includes chapters on detoxication; urine formation, composition and excretion; amniotic and allantoic fluids; the perspiration; and intestinal excretion.

The final section is headed "Nutritive Exchanges" and has three chapters concerned with, respectively, the foodstuffs, energy exchanges and exchanges of materials.

The book is written in an admittedly didactic style, with no bibliography or references to the literature as supporting material for statements which are made. It is particularly regrettable that no bibliography or selected readings are presented. Nevertheless, the book is surprisingly up-to-date in its information, especially if one considers the difficulties in securing literature published during the war years. Certain

of the most recent advances in intermediary metabolism are found mentioned in more or less detail. One might take exception to the allocation of space given to certain topics. However, the solution of this problem invariably reflects the personal interests and points of view of the authors, in addition to the actual importance of the subject matter. Perhaps the reviewer is unnecessarily agitated by the fact that only 11 lines are devoted to the hormones of the hypophysis, while in a textbook ostensibly for students of medicine, plant hormones are given space of slightly more than one page.

Despite the didactic nature of the presentation, which borders frequently on undue brevity, a number of subjects, including newer concepts, that are not commonly found in textbooks, are present in this volume. For example, the following topics are referred to briefly, or, in certain instances, discussed at greater length: the chlorophylls; composition of vitreous humor and of seminal fluid; oxidation-reduction potential; utilization of isotopes in studies of metabolism; amniotic and allantoic fluids.

In the reviewer's opinion, this volume represents one of the most complete and valuable textbooks available in the field of general biochemistry. It is comprehensive in its scope, although, as indicated, some topics are given limited consideration. If it were available in English, the book would undoubtedly find wide use as a basic textbook by students and teachers of biochemistry in this country.

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**Vitamins and Hormones. Vol. VII.** Edited by ROBERT S. HARRIS, Massachusetts Institute of Technology, and KENNETH V. THIMANN, Harvard University, Cambridge, Massachusetts. Academic Press, Inc., New York, N. Y., 1949. xii + 488 pp. Price \$7.80.

The editors of this volume have produced again a well-balanced group of reviews on vitamins and hormones which are integrated with those published in the six previous volumes. For example, this volume contains a chapter on the nutritional requirements of rats by R. A. Brown and M. Sturtevant and one on the nutritional requirements of guinea pigs by G. J. Mannering; these complete a group of articles dealing with the requirements of the more important laboratory animals started in Vol. II.

I doubt that the editors could have found authors better qualified to prepare the specific reviews than those who have prepared the articles for this volume. Zechmeister, who has spent much of his lifetime working on carotenoids, reviews "Stereoisomeric Provitamins A." B. C. P. Jansen, who with Donath first obtained the antineuritic vitamin in crystalline form in 1926, gives an excellent summary of the physiology of thiamine. W. A. Krehl, who did much of the early work on the relation of tryptophan and niacin, covers "Niacin in Amino Acid Metabolism." Clive M. McCay reviews diet and aging and I suppose no one has given more attention to this subject than he.

The very complete review of vitamin P by Scarborough and Bacharach is especially timely when attempts are being made to determine if the use of the term vitamin P should be continued since, as the authors point out, the compounds included under the term are not closely related chemically and have been used at levels which suggest pharmacological action.

The chemistry of the gonadotropic hormones and of luteotropin (prolactin) is covered by active workers in these fields, C. H. Li and Abraham White, respectively.

R. N. Jones and K. Dobriner give extensive data on the infrared spectrometry of the steroids much of which comprises previously unpublished material.

Although alloxan diabetes is a relatively new subject, many papers dealing with this problem have been published and C. C. Bailey reviews most of them in his chapter.

The last two chapters deal with the hormones of the posterior lobe of the pituitary gland. Both chapters have been prepared by R. L. Stehle. In the first one he brings previous reviews on the chemistry of these hormones up to date, and in the second he discusses the action of these hormones upon circulation and the secretion of urine.

Certainly this volume, together with the previous six, will be of most value to the active research worker as well as those who cover vitamins and hormones in their teaching.

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# The Antagonism of Sodium Tripolyphosphate and Adenosine Triphosphate in Yeast

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## INTRODUCTION

In 1938 and 1943 Neuberg and co-workers (1, 2) described sodium tripolyphosphate ( $\text{Na}_5\text{P}_3\text{O}_{10}$ ) and its decomposition in contact with many different tissues. It is of interest, therefore, to determine its physiological activity. A preliminary observation indicated that upon the addition of triphosphate to yeast the rate of fermentation of glucose was lowered. The following experiments indicate that triphosphate inhibits yeast hexokinase, and that this inhibition is reversed by adenosine triphosphate (ATP) or by magnesium.

## MATERIALS AND METHODS

*Saccharomyces cerevisiae*, Anheuser-Busch strain HG, was isolated from uncontaminated beer sludge and cultured on the following medium:

Difco yeast extract	2.0 g.
Bacto tryptone	5.0 g.
$(\text{NH}_4)_2\text{SO}_4$	1.0 g.
$\text{KH}_2\text{PO}_4$	2.0 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.25 g.
$\text{CaCl}_2$	0.13 g.
Na-lactate (70% wt./vol.)	5.0 g.
Glucose	40.0 g.
$\text{H}_2\text{O}$	1000 ml.

Cultures were incubated at 30.5°C. and used after 40–48 hr. At that time the cells contained little stored glycogen, as determined by iodine staining (3). For experiments

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a 50-ml. culture was centrifuged, washed 3 times with  $M/15$  phosphate buffer of the appropriate pH, and resuspended in phosphate buffer. The density of the suspension was adjusted to 3.0 mg./ml. dry weight with the aid of a Klett-Summerson colorimeter.

The method of Folin and Malmros, as described by Umbreit *et al.* (4), was employed to determine glucose. The color developed was measured in a Coleman spectrophotometer at 520  $m\mu$ .

Sodium tripolyphosphate was obtained through the courtesy of Monsanto Chemical Co., Phosphate Division, St. Louis, Mo., and was purified in our laboratory by recrystallization from water and ethanol. The crude and purified preparations both exhibited the same properties. In distilled water the pH of a triphosphate solution is 9.8; sufficient  $H_3PO_4$  was added in each experiment to adjust the pH to 7.6 (pH 4.5 for the experiment described in Table I).

For ymase preparations, dried yeast was ground for 10 hr. in a ball mill at 5°C., extracted for 2 hr. with 3 parts  $M/15$  pH 7.0 phosphate buffer at 35°C., diluted 5 times with fresh buffer, and centrifuged. The clear supernatant liquid fermented glucose. Crystalline yeast hexokinase was obtained through the courtesy of Dr. S. Colowick. The enzyme was stabilized in solution by the addition of 5 parts of egg albumen to 1 part of hexokinase. Its activity was measured manometrically according to Colowick and Kalckar (5). For one experiment Dr. G. Pinchot kindly supplied a purified hexokinase preparation.

All experiments were performed in a Warburg respirometer at 30.2°C.; the vessels oscillating 110 times/min. through an arc of 5 cm.

## EXPERIMENTAL RESULTS AND DISCUSSION

Addition of varying amounts of triphosphate to yeast fermenting glucose led to a depression of  $CO_2$  production. The experiment is described in Table I. Although the decrease in  $CO_2$  output in the presence of triphosphate was definite, the effect was not lasting: it disappeared in the vessel with the lowest triphosphate concentration after 10 min., and in the vessel with the highest concentration after 30 min. After that time fermentation in all 5 vessels proceeded at the same rate. It may be concluded that the triphosphate inhibition was being removed, and that the time of removal varied directly with the amount of triphosphate initially present. Triphosphate, it was found later, is rapidly hydrolyzed by yeast in acid media; hence the rapid disappearance of triphosphate inhibition mentioned earlier. All subsequent experiments were therefore performed at pH 7.6, where triphosphate disappeared only slowly. Apparently the triphosphatase present in yeast (2) is less active at this hydrogen-ion concentration. An experiment at pH 7.6 gave the results shown in Table II.

It appears likely, therefore, that triphosphate retards glycolysis, and its structure suggests that triphosphate may compete with ATP in one

TABLE I

*Effect of Triphosphate on the CO<sub>2</sub> Output of Fermenting Yeast I*

Contents of vessels: *Side arms* 0.2 ml. 1% glucose, 0.02, 0.04, 0.1, 0.2 ml. 1M triphosphate, respectively. *Main vessel* 2.0 ml. yeast suspension (3.0 mg. ml.) in *M* 15 pH 4.5 phosphate buffer. Atmosphere: 95% N<sub>2</sub>, 5% CO<sub>2</sub>. Total volume: 2.7 ml. Time: 10 min.

Vessel	Triphosphate	CO <sub>2</sub> during first 10 min.	Per cent of control
	<i>μmoles</i>	<i>μl.</i>	
1	0	125	100
2	20	117	94
3	40	101	81
4	100	93	74
5	200	83	67

or both reactions in which ATP enters the glycolytic cycle. The hypothesis of competitive inhibition was first tested in yeast zymase preparations. Triphosphate slowed the anaerobic dissimilation of glucose by zymase, as indicated by determinations of residual glucose. The inhibition was diminished as the concentration of ATP was increased (Table III). This competition could be shown more clearly by the use of crystalline yeast hexokinase. In contrast to intact yeast cells or zymase preparations, the activity of pure hexokinase could be completely inhibited by triphosphate (Table IV). Here again the inhibition was overcome by adding excess ATP to the reaction mixture. Hexokinase activity returned at higher ATP concentrations in the presence of a constant triphosphate level (Table V).

TABLE II

*Effect of Triphosphate on the CO<sub>2</sub> Output of Fermenting Yeast II*

Contents of vessels: *1st side arm* 0.2 ml. 1% glucose, 0.25 ml. 1M triphosphate; *2nd side arm* 0.2 ml. 2 N H<sub>2</sub>SO<sub>4</sub>. *Main vessel* 2.0 ml. yeast suspension (3.0 mg./ml.) in *M*/15 pH 7.6 phosphate buffer. Atmosphere: 95% N<sub>2</sub>, 5% CO<sub>2</sub>. Total volume: 2.7 ml. Time: 10 min.

Vessel	Triphosphate	CO <sub>2</sub> during first 10 min. (including HCO <sub>3</sub> <sup>-</sup> formed)	Per cent of control
	<i>μmoles</i>	<i>μl.</i>	
1	0	174	100
2	250	25	14

TABLE III

*Competition of Triphosphate and ATP in Yeast Zymase Preparation*

Contents of vessels: *Side arm* 0.35 ml. 1% glucose; 0.1 ml. 1 *M* triphosphate where indicated; 0.1, 0.2, 0.3, 0.4 ml. 0.15 *M* ATP, respectively. *Main vessel* 2.0 ml. yeast zymase preparation in *M*/15 pH 7.5 phosphate buffer; 0.2 ml. 0.1 *M* MgSO<sub>4</sub>. Atmosphere: 95% N<sub>2</sub>, 5% CO<sub>2</sub>. Total volume: 3.5 ml. Time: 30 min.

Vessel	Triphosphate	ATP	Glucose consumed in 30 min.	Per cent of control
	<i>μmoles</i>	<i>μmoles</i>	<i>mg.</i>	
1	0	15	1.61	100
2	100	15	0.28	17
3	100	30	0.45	28
4	100	45	1.25	78
5	100	60	1.35	84

At least two hypotheses can account for the antagonism between triphosphate and ATP. These compounds may compete for hexokinase, or, both being strong metal-binding agents (6, 7), they compete for the magnesium essential for hexokinase activity. Table VI shows reversal of triphosphate inhibition by increased magnesium concentrations. Triphosphate, therefore, inhibits the hexokinase reaction by binding magnesium in a soluble undissociated complex. Since ATP, too, is a chelating compound (7), it can compete with triphosphate for metals,

TABLE IV

*Effect of Triphosphate on Crystalline Hexokinase*

Contents of vessels: *Side arm* 0.5 ml. 0.016 *M* ATP; 0.1 ml. 0.1 *M* NaHCO<sub>3</sub>. *Main vessel* 0.03 ml. 0.1% hexokinase in 0.5% egg albumen; 0.2 ml. 0.1 *M* MgSO<sub>4</sub>; 0.1 ml. 0.5 *M* glucose; 0.5 ml. 0.1 *M* NaHCO<sub>3</sub>; 0.01, 0.03, 0.1 ml. 0.1 *M* triphosphate, and 0.03, 0.1, 0.2 ml. 1 *M* triphosphate, respectively. pH: 7.2. Atmosphere: 95% N<sub>2</sub>, 5% CO<sub>2</sub>. Total volume: 2.13 ml. Time: 15 min.

Vessel	Triphosphate	CO <sub>2</sub> in 15 min.	Per cent of control
	<i>μmoles</i>	<i>μl.</i>	
1	0	175	100
2	1	152	87
3	3	120	69
4	10	45	26
5	30	12	7
6	100	3	2
7	200	0	0

and reverse triphosphate inhibition by binding enough magnesium for the hexokinase reaction to occur. These experiments suggest that magnesium may be bound in the hexokinase reaction complex by virtue of the chelating properties of ATP.

TABLE V

*Reversal of Triphosphate Inhibition by ATP in a Crystalline Hexokinase System*

Contents of vessels: *Side arm* 0.1 ml. 0.1 *M* NaHCO<sub>3</sub>; 0.03, 0.06, 0.1 ml. 0.016 *M* ATP, respectively. *Main vessel* 0.03 ml. 0.1% hexokinase in 0.5% egg albumen; 0.2 ml. 0.1 *M* MgSO<sub>4</sub>; 0.1 ml. 0.5 *M* glucose; 0.5 ml. 0.1 *M* NaHCO<sub>3</sub>; 0.1 ml. 0.1 *M* triphosphate where indicated. pH: 7.2. Atmosphere: 95% N<sub>2</sub>, 5% CO<sub>2</sub>. Total volume: 2.13 ml. Time: 15 min.

Vessel	Triphosphate	ATP	CO <sub>2</sub> in 15 min.	Per cent of control
	<i>μmoles</i>	<i>μmoles</i>	<i>μl.</i>	
1	0	4.8	105	100
2	10	4.8	27	26
3	10	9.6	46	44
4	10	16.0	91	87

Sodium tripolyphosphate did not affect the aerobic dissimilation of glucose by intact cells either of *S. cerevisiae* HG or of strictly aerobic wild yeasts, although under aerobic conditions the phosphate turnover per mol of glucose is 6–8 times greater than during fermentation. It is

TABLE VI

*Reversal of Triphosphate Inhibition by Mg in a Purified Hexokinase System*

Contents of vessels: *Side arm* 0.01 ml. purified hexokinase preparation; 0.1 ml. 0.1 *M* NaHCO<sub>3</sub>; 0.2 ml. 0.04 *M* ATP. *Main vessel* 0.1, 0.2, 0.4 ml. 0.1 *M* MgSO<sub>4</sub>, and 0.1 ml. 1 *M* MgSO<sub>4</sub>, respectively; 0.5 ml. 0.1 *M* NaHCO<sub>3</sub>; 0.1 ml. 0.5 *M* glucose; 0.2 ml. 0.01 *M* NaF; 0.1 ml. 0.4 *M* triphosphate where indicated. pH: 7.2. Atmosphere: 95% N<sub>2</sub>, 5% CO<sub>2</sub>. Total volume: 2.2 ml. Time: 10 min.

Vessel	Triphosphate	MgSO <sub>4</sub>	CO <sub>2</sub> in 10 min.	Per cent of control
	<i>μmoles</i>	<i>μmoles</i>	<i>μl</i>	
1	0	10	142	100
2	40	10	26	18
3	40	20	45	32
4	40	40	67	47
5	40	100 <sup>a</sup>	101	72

<sup>a</sup> Precipitate of MgHPO<sub>4</sub> in vessel.

not clear, however, whether this observation casts doubts on the role of the hexokinase reaction in glucose oxidation.

### SUMMARY

Sodium tripolyphosphate ( $\text{Na}_5\text{P}_3\text{O}_{10}$ ) inhibits the anaerobic conversion of glucose by intact yeast cells, yeast zymase preparations, or crystalline hexokinase. This inhibition can be removed either by adenosine triphosphate (ATP) or by magnesium. It is suggested that magnesium may be bound in the hexokinase reaction complex by virtue of the chelating properties of ATP.

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# The Metabolism of *p*-Aminobenzoic Acid in the Rabbit

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## INTRODUCTION

In view of the limited data relating to the conjugation of *p*-aminobenzoic acid (PABA) with acetyl groups and with glucuronic acid (1, 2), particularly in the rabbit, we have carried out experiments with this species in which the  $\alpha$ -naphthol procedure for the determination of glucuronic acid has been employed. Griffith (3) observed that conjugation with both glycine and glucuronic acid was important in the detoxication of benzoic acid in the rabbit, and that administration of glycine with the benzoic acid tended to depress conjugation with glucuronic acid. A similar reciprocal relation between the conjugation of benzoic acid with glycine and with glucuronic acid has been observed in the pig (4). We were therefore interested in studying the relationship between acetylation and glucuronic synthesis after the administration of PABA. What influence does the administration of potential acetyl group precursors have upon the conjugation of PABA with glucuronic acid?

## EXPERIMENTAL

Male rabbits, 2-3 kg. in weight, maintained on a diet of cabbage and oats served as subjects (5). After fasts of 18 hr. prior to feeding, urine samples were collected in 6- and 18-hr. periods daily and the excretions of PABA are reported as values for 6 and 24 hr. respectively. The sodium salt of PABA was fed by stomach tube at the level of 200 mg. of the acid/kg.; glycine, in amounts equivalent to 3 moles of the PABA administered; and DL-malic acid, equivalent to 10-20 molar equivalents as indicated in the table.

The free arylamino group of PABA reacts with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) to give a lemon yellow color. The details of the reaction as applied

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<sup>2</sup> Government of India Scholar.



to the determination of *p*-aminosalicylic acid have been described (6). This procedure has been followed in detail in the present studies. All measurements were made with an Evelyn photoelectric colorimeter (filter No. 470). A standard curve, prepared with concentrations of PABA ranging from 4–30  $\mu\text{g.}$ , was a straight line, when  $\log I_0/I$  was plotted against concentration. Acetylated PABA was calculated as the difference between the free acid and the total PABA as determined after hydrolysis with *p*-toluenesulfonic acid for 3 hr. at 100°C. (6). Although these appeared to be the optimal conditions for the hydrolysis, incomplete recoveries were obtained in control experiments. Losses appeared to be due primarily to destruction of PABA, free or liberated in the hydrolysis (7). In control experiments, determinations run under conditions similar to those encountered in the experimental urines, in which amounts of free and acetylated PABA ranging from 0–32  $\mu\text{g.}$  were added to diluted normal rabbit urine, the recovery of PABA after hydrolysis varied from 81–89%, with an average value of 84%. Doisy and Westerfield (7), who used a slightly different procedure for hydrolysis, reported an average loss of 6%. We have multiplied the values of total PABA obtained in the experimental urines by the factor 1.19 (100/84) to obtain the corrected amounts of PABA.

Glucuronic acid was estimated by the modification of the method of Maughan, Evelyn, and Braun as used in this laboratory (5).

## DISCUSSION

As shown in Table I, 68–96% of the PABA fed was excreted in the urine within 24 hr., and of the total PABA excreted, 42–50% was present as the acetylated form in control experiments in which no supplement was fed with the PABA. More than half of the total amount of acetylated PABA excreted in the 24-hr. period was present in the urine collected in the first 6-hr. period in the control experiments and in those in which glycine was administered. On the other hand, there appeared to be a delay in either acetylation or excretion of the acetylated derivative when DL-malic acid was fed, since, of the total PABA excreted in acetylated form in 24 hr., only 22–44% was excreted in the urine of the 6-hr. period. The excretion of the acetylated PABA, however, continued during the succeeding 18-hr. period, so that the total excretion of this derivative of PABA in 24 hrs. was equal to or greater than that in the other experiments.

The degree of acetylation in our control experiments is greater than that reported by Bray (2) who, with dosages of PABA comparable to those used by us, observed an excretion of only 4–21% of the total amount fed as the acetylated form. It may be pointed out that these workers made no correction for any destruction of PABA during acid hydrolysis. This difference alone can hardly account for the higher percentage of acetylation observed in these experiments.

When glycine was fed with the PABA, the excretion of acetylated derivative in both the 6- and 24-hr. periods was increased. This is evident when the values are calculated either as per cent of the total amount administered (columns 4 and 7 of Table I) or of the total PABA excreted (columns 5 and 8). Similarly when malic acid was fed, a considerable increase in acetylation was observed in the 24-hr. period.

TABLE I

*Urinary Excretion of Free and Acetylated p-Aminobenzoic Acid (PABA) and of Glucuronic Acid after Oral Administration of PABA*

In column 1, the first digit is the number of the experiment and the second, the number of the experimental animal. Glucuronic acid is calculated as glucurone. In the last 2 columns, the values are calculated on the assumption that 1 mole of PABA conjugates with 1 mole of glucuronic acid.

Expt. no.	Supplement fed	PABA						Glucuronic acid		
		6 hr.			24 hr.			24 hr.		
		Total	Acetylated		Total	Acetylated		Extra		
		Of intake	Of intake	Of total excreted	Of intake	Of intake	Of total excreted	Of PABA intake		Of free PABA excreted
		per cent	per cent	per cent	per cent	per cent	per cent	mg.	per cent	per cent
1-7	None	68	25	37	82	36	44	233	26	59
4-7	None	59	19	32	74	31	42	186	21	52
3-7	None	64	22	34	79	33	42	192	22	52
5-7	Glycine	62	31	50	80	44	55	45	6	17
9-7	Glycine	73	39	53	90	52	58	52	6	18
14-7	Malic acid	52	24	46	93	55	59	56	10	27
6-2	None	56	24	43	96	46	48	100	15	31
7-2	None	54	26	48	91	44	48	106	13	34
8-2	Glycine	68	38	56	90	55	61	73	9	33
10-2	Malic acid	40	16	40	83	47	57	51	9	25
17-3	None	56	23	42	68	33	49	112	13	62
18-3	None	39	15	38	79	39	50	120	14	59
13-3	Glycine	59	35	59	84	56	67	73	9	33
11-3	Malic acid	38	25	66	84	67	80	45	6	41
12-3*	Malic acid	23	13	56	72	58	81	Not determined		

\* Four g./kg. body weight (20 equiv.) were fed in this experiment. Two g. kg. in other malic acid experiments.

an increase which was particularly marked with Rabbit 3. It may be noted that malic acid when fed in smaller amounts of 1 g./kg. (5 equiv. approximately) did not influence the degree of acetylation of PABA.

The excretions of "extra" glucuronic acid (5) after oral administration of PABA are shown in the last three columns of Table I. Bray (2) observed an excretion of glucuronic acid equivalent to 8-24% of the PABA fed. We have obtained similar values in control experiments, the extra glucuronic acid corresponding to 13-26% of the amount fed. When glycine or malic acid was fed with the PABA, the excretion of glucuronic acid, presumably in conjugation with PABA as the ester type and unstable in alkali, was depressed both in absolute amount and in percentage of the PABA fed. Since the administration of these supplements was associated with an increased excretion of acetylated derivatives, it may be conjectured that the reactions which result in acetylation in some way depress the formation and excretion of the conjugated glucuronides. Since the mode of synthesis and the fate of glucuronic acid in the animal body have not been explained clearly as yet, further experiments are necessary.

The PABA conjugated with glucuronic acid would be determined in the free PABA fraction since the arylamino group is uncombined. No attempt has been made to distinguish between unconjugated ("free") PABA and the ester type of PABA glucuronide in this reaction with Ehrlich's reagent. We have made no attempt to determine the presence of a glycine conjugate in our experimental urines, since we know of no evidence of such a conjugation *in vivo* (1, 2).

### SUMMARY

1. When moderate amounts (0.20 g./kg.) of *p*-aminobenzoic acid (PABA) were fed to rabbits, the excretions of both *p*-acetylaminobenzoic acid and extra glucuronic acid indicated that both these conjugations were utilized by this species in the metabolism of PABA.

2. When glycine or DL-malic acid was fed with the PABA, an increased excretion of the acetylated derivative was observed, suggesting that these two compounds were effective sources of groups for acetylation.

3. When the excretion of acetylated PABA was increased by the simultaneous administration of glycine or malic acid and PABA, a marked decrease in the excretion of extra glucuronic acid was observed

as compared with the values obtained when PABA was fed without a supplement. It is suggested that the reactions associated with acetylation depress the synthesis of or conjugation with glucuronic acid.

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# The Biochemistry of Rubber Formation in the Guayule.

## II. Rubber Formation in Aseptic Tissue Cultures<sup>1</sup>

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### INTRODUCTION

In an earlier paper of this series, the biochemistry of rubber synthesis in intact seedling guayule plants has been discussed. It was shown that with such seedling plants, grown in solution cultures, it is possible to increase the amount of rubber accumulated by the application of various substances, possible intermediates in rubber synthesis, to the nutrient solution in which the seedlings are grown. Substances active in increasing rubber formation in such seedlings were found to be acetate, acetone, acetoacetate, glycerol, and  $\beta$ -methylcrotonic acid. In the guayule, rubber is found and is produced primarily in the bark of the stems and roots and but little rubber is produced in the leaf tissue, a fact which suggests at once that it would be of interest to study rubber synthesis and accumulation in isolated stem or root tissues. Such a study is the more of interest since it is known (1) that the accumulation of rubber in the stem and roots of the guayule depends closely on the presence of actively metabolizing leaves. When the leaves are removed rubber formation in the stem and roots essentially ceases. By the use of a technique for culture of isolated segments of stem it has been possible to study this interrelation of stem and leaves in the formation of rubber. The present paper describes a method for the study of

<sup>1</sup> Report of work carried out as a cooperative project between the California Institute of Technology and the U. S. Department of Agriculture, Bureau of Plant Industry, Soils and Agricultural Engineering, Division of Rubber Plant Investigations.

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rubber production by isolated segments of guayule stem tissue, and describes further the effects on rubber synthesis obtained by the addition of leaf extracts and of various pure compounds to the nutrient solutions in which such stem tissues were grown.

## METHODS

The tissue used for these experiments consisted of segments of guayule stems taken from plants grown in the greenhouse under conditions of relatively high temperature (minimum night temperature 21°C.) and containing only low concentrations of rubber (0.1–0.3%). The cuttings were obtained from approximately 1-yr.-old plants grown in sand culture supplied with complete nutrient solution. The branches selected were of the current season's growth and of a diameter of approximately 5 mm. The general technique for the preparation of the cuttings was to remove sections of stem, approximately 12–15 cm. long. These stems were then defoliated and superficially sterilized by immersion in 2% sodium hypochlorite solution for a period of 20 min. The stem pieces were next washed several times with sterile glass-distilled water and the epidermis was then carefully peeled from the entire stem with a scalpel. These manipulations as well as those that follow were all carried out in an aseptic tissue-culture transfer-room. After the epidermis had been peeled from the section of stem, the stem was divided into pieces approximately 1.5–2 cm. long. The sections from any one piece of stem were divided between the different treatments in any one experiment so that the 20 individual cuttings present in a particular treatment of an experiment represented cuttings taken from 20 different stems. The stem cuttings prepared in this way were placed individually upon the surface of agar slants contained in 25 × 150-mm. pyrex culture tubes.

The basal medium used for the culture of the stem cuttings is given in Table I. This medium includes inorganic salts, identical with those used in this laboratory for root culture; sucrose, as a source of carbohydrate; and the accessory growth factors, thiamine, pyridoxin, nicotinic acid, and yeast extract. The accessory growth factors were found to increase growth somewhat above the level which would obtain in the

TABLE I  
*Composition of the Basal Medium Used for Growing Aseptic Guayule Cuttings*

Component	Amount	Component	Amount
			mg.
Glass-dist. H <sub>2</sub> O	1 l.	HCl	65
Sucrose	20 g.	KH <sub>2</sub> PO <sub>4</sub>	20
Agar	15 g.	Ferric tartrate	1.5
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	236 mg.	Vitamin B <sub>1</sub> , B <sub>6</sub> , nicotinic acid, each	0.5
MgSO <sub>4</sub> ·7H <sub>2</sub> O	36 mg.	Yeast extract	100
KNO <sub>3</sub>	81 mg.	IAA	10

medium without these addenda. Indoleacetic acid was further added to the nutrient solution in order to bring about proliferation of callus tissue, as will be discussed below. Ten ml. of medium was placed in each culture tube, and 20 cultures were used in each experimental treatment. The stem cultures were incubated in conditions of constant temperature, 27°C., under fluorescent lights supplying an intensity of approximately 400 foot-candles. They were thus maintained for a period of 3-7 weeks, and were then harvested, the contaminated cultures discarded, and the entire lot of cuttings from each treatment dried at 70°C., ground to 40-mesh and analyzed for rubber.<sup>3</sup> The methods of analysis used in the present work were the same as those outlined in the earlier paper (2). These include determination of rubber by a colorimetric method which involves preliminary extraction of the ground sample with acetone to remove resins and other interfering materials, extraction of the rubber with benzene, and precipitation of the rubber from the benzene solution by methyl alcohol. The rubber is then determined turbidimetrically. In addition to this determination, total rubber was also usually determined by elimination of the preliminary acetone treatment. It was found that with cuttings as with the seedlings described earlier much of the rubber which is formed during the experimental period appears to be slightly acetone-soluble and is in part removed by the acetone pre-extraction. This rubber has been tentatively referred to as "low-molecular-weight rubber." As a final check upon the quantities of rubber produced in the different treatments, resort was frequently had to the microbromination method of Willits, Swain, and Ogg (4).

### *Variability of Tissue Culture Material*

The variability in growth and rubber concentration found in replicate lots of sterile guayule stem cuttings was determined in a series of experiments of which typical results are given in Table II. In the experiment of Table II it was found that the average initial dry weight per cutting was 20.5 mg. and that the tissue contained 0.10% of total rubber. After 7 weeks of incubation, the cultures had increased in dry weight to a total of approximately 38 mg. per cutting or almost double the initial weight. The increments in dry weight differ by an average of less than 10% from the mean increment. Table II shows also that the concentration of rubber found in the tissues did not change during the culture period, and that the concentration of rubber found is highly reproducible.

### *Influence of Indoleacetic Acid on the Growth of the Cuttings*

When guayule stem cuttings prepared as described above are placed on basal nutrient without indoleacetic acid, little growth occurs, as is shown in Table III. If indoleacetic acid is added to the nutrient solution

<sup>3</sup> All analyses reported here were carried out by Betty Jean Wood.

TABLE II

*Variability in Dry Weight and Rubber Formation by Aseptic Guayule Cuttings*

Replicate no.	Average dry wt./cutting mg.	Per cent of rubber; dry weight basis
Initial	20.5	0.10
1	37.1	0.09
2	38.1	0.10
3	41.2	0.10
4	37.8	0.10

at a concentration of 1 mg./l., a moderate growth of callus tissue takes place during the incubation period. This callus is produced both at the basal end of the cutting and along the zone of contact between the cutting and the medium. When indoleacetic acid is added to the nutrient solution in a concentration of 10 mg./l., the growth of callus tissue is still more extensive, the growth taking place not only on the surface of the cutting but also internally and resulting in swelling of the stem tissue. The use of indoleacetic acid in still higher concentrations does not further increase the amount of callus produced, and the increase in dry weight during the incubation period is also not greater than the level attained with 10 mg. of indoleacetic acid/l. The callus tissue produced during growth of the stem sections consists mainly of undifferentiated parenchymatous cells which, as will be shown below, are potentially capable of rubber accumulation. Attempts were made to isolate and culture the callus tissue but all trials were unsuccessful.

Table III shows, as does Table II, that the rubber concentration found in the tissues during growth under the influence of indoleacetic acid remains unchanged. It would appear, therefore, that during cul-

TABLE III

*Effect of Varying Concentrations of I.A.A. on the Dry Weight and Rubber Content of Guayule Cuttings*

Treatment	IAA in basal medium	Average dry wt. per cutting	Per cent of rubber; dry wt. basis
	mg./l.	mg.	
Initial	—	22.5	0.12
1	None	27.8	0.12
2	1	37.4	0.12
3	10	37.8	0.12
4	100	31.2	0.11



ture and growth the isolated stem tissue is able to produce a small amount of rubber and thus to maintain a constant rubber concentration. It is, however, unable to produce a sufficient amount of rubber to increase the rubber concentration under the present experimental conditions. This fact implies that the sugar and other nutrients which suffice for growth of the tissue do not support extensive rubber synthesis.

#### *Influence of Leaf Extracts in Rubber Formation*

Rubber formation in excised stem sections grown in culture may be induced by suitable extracts prepared from leaves of guayule plants which are actively engaged in rubber accumulation, as is shown in Table IV. The data in this table were obtained from experiments in which guayule stem sections were cultured on basal nutrient to which was added the acetone extract of guayule leaves. The leaves used for the experiment were obtained from plants grown under conditions of low temperature, which is known to favor rubber synthesis (1), and the plants were shown analytically to be actively engaged in rubber accumulation. These leaves were ground in a Waring blender at 0°C. and were then extracted with acetone in a Soxhlet extractor. The extract was filtered, the acetone removed under reduced pressure, and the residue taken up in water. Aliquots of this extract were added to each test tube before sterilization. Table IV shows that when such extracts were added to the nutrient, rubber formation in the excised stem tissue was increased markedly. It would appear therefore that the leaf extract contains either (a) a material which can act as a substrate for rubber formation by the stem tissue, or (b) a material which in some way stimulates the rubber-forming system of the isolated stem tissue. Table IV also includes results obtained when the extract of leaves of guayule plants grown under warm temperature conditions and not actively engaged in rubber accumulation was added to the nutrient solution in which tissue cultures were grown. Extracts of such leaves appear to be inactive in supporting rubber formation in excised stem tissue. Attempts were made to purify and concentrate the active principle of guayule leaf extract. The active material may consist of more than one compound, as is shown by the fact that when the active aqueous extract was steam distilled from acid solution both the distillate and the nonvolatile residue were found to support rubber formation in excised stem cuttings.

TABLE IV

*Effect of Acetone Extracts of Guayule Leaves on Rubber Formation  
by Aseptic Excised Stem Sections of Guayule*

Addenda to nutrient	Per cent rubber in sections	
	Ext. made from plants accumulating rubber	Ext. made from plants not accumulating rubber
None (control)	0.06	0.07
Ext. of 2.6 mg. leaf/ml.	0.63	0.07
Ext. of 5.2 mg. leaf/ml.	1.21	0.07
Ext. of 10.4 mg. leaf/ml.	0.22	0.07

No considerable success has as yet been obtained in elucidation of the nature of the principle promoting rubber formation in the extract of guayule leaves. It is clear however that leaves of plants actively engaged in rubber synthesis do exert an effect on rubber formation in isolated stem tissues, and that this factor is absent (or present to a lesser extent) in leaves of plants not engaged in active rubber accumulation.

#### *Influence of Various Pure Substances on Rubber Formation*

A variety of pure substances were tested for possible activity in supporting rubber formation in isolated guayule stem cultures. These substances were chosen partially on the basis of their structural relationships to isoprene or isoprenoid compounds. With few exceptions the substances tested were totally inactive on rubber formation in guayule stem cuttings under the conditions of the present experiments. The substances found to be inactive and the concentration ranges tested are listed in Table V. Of all substances investigated in the present work, only three yielded results indicating definite ability to support increased rubber formation in isolated stem tissue. These substances were acetate, acetone, and  $\beta$ -methylcrotonic acid.<sup>4</sup>

#### *Effects of Acetate and Its Presumed Derivatives on Rubber Formation*

Table VI gives results of a typical experiment in which acetate was added as sodium acetate in a concentration of 100 mg. 'l. (a concentration previously found to be optimal) to the nutrient solution in which guayule stem tissues were cultured. Table VI shows that this concentration of acetate resulted in an increase in rubber concentration in the

<sup>4</sup> Prepared according to the method outlined in Ref. (3).

TABLE V

*Substances Found Ineffective in Supporting Increased Rubber Formation in Isolated Guayule Cuttings*

Substance	Concentration in g./liter of medium	Substance	Concentration in g./liter of medium
	g./l.		g./l.
Crotonaldehyde	0.01	L-Leucine	0.10
Tiglaldehyde	1.00	DL-Leucine	0.10
Isovaleraldehyde	0.01	Glutamic acid	0.10
Pyruvic acid	0.10	L-Arabinose	0.10
Tiglic acid	0.10	L-Sorbose	0.10
Citraconic acid	0.10	$\alpha$ -Methylglucoside	0.10
Itaconic acid	0.02	Acetone + acetaldehyde	0.10-1.0
4-Pentenoic acid	0.20	Glycerol + pyruvic acid	0.10-1.0
$\alpha$ -Ketoglutaric acid	0.10		
Glycerol	1.0-5.0		
D-Valine	0.10		

stem tissue of over 100% above that found in the control tissue cultured under similar conditions but in a medium lacking added acetate. The effect of acetate in promoting increased rubber accumulation was found and confirmed in a total of 9 experiments. Additions of acetone to the nutrient solution used for culture of excised guayule stem sections also resulted in increased rubber deposition. The concentration of acetone used in the experiment of Table VI was 1 g./l., a concentration previously found to be optimal, and the acetone was added aseptically to the nutrient solution after sterilization of the nutrient. Acetone, like acetate, brought about an increase of over 100% in the concentration

TABLE VI

*Effect of Acetate and Acetone on the Formation of Rubber by Aseptic Guayule Cuttings*

Treatment no	Addenda	Average dry wt. per cutting	Per cent rubber, dry wt basis
		mg.	
1	Control	34.6	0.15
2	Acetone 1 g./l.	35.2	0.34
3	Na acetate 100 mg./l.	—	0.32
4	Na acetate 100 mg./l. + acetone 1 g./l.	27.3	0.44

of rubber found in the experimental tissue. When acetate and acetone were supplied simultaneously in the nutrient solution the increase in rubber accumulated was above the level brought about by either material alone, as is also shown in Table VI. In this case the final concentration of rubber found in the stem tissues was approximately three times that found in the control tissues. The effects of acetone, like those of acetate, have been confirmed in repeated experiments.

TABLE VII

*Effect of  $\beta$ -Methylcrotonic Acid on the Production of Rubber by Excised Guayule Stem Cultures*

Rubber determined gravimetrically as crystalline rubber tetrabromide.

Treatment	Addenda	Rubber: per cent of dry weight
1	None (control)	0.27
2	$\beta$ -Methylcrotonic acid 20 mg./l.	0.85
3	$\beta$ -Methylcrotonic acid 100 mg./l.	0.36

The third substance capable of supporting increased rubber synthesis in the cultures was  $\beta$ -methylcrotonic acid, a material previously reported (2) to be effective in increasing rubber accumulation in guayule seedlings. Table VII shows that this substance, like acetate and acetone, markedly increased rubber deposition when it was supplied to the nutrient solution used for growth of aseptic guayule cuttings.

## DISCUSSION

Although rubber is accumulated in the parenchymatous cells of root and stem of the guayule, it is known (1) from defoliation experiments that the leaves play an important and immediate role in the accumulation process. With the present technique of culture of isolated stem fragments, it is possible to demonstrate directly the interrelation of stem and leaves in rubber formation. Isolated leafless stem fragments make a vigorous callus growth *in vitro* when provided with a nutrient solution containing mineral salts and sucrose as the major constituents. Despite this growth, such sections form only minute amounts of rubber and do not increase in rubber concentration during the culture period. That the tissue is capable of rubber accumulation is shown by the fact that extensive rubber formation can be induced *in vitro* by the addition to the nutrient of an extract of the leaves of plants which are themselves rapidly synthesizing rubber. These experiments suggest then that the leaves of guayule plants which are engaged in rubber ac-

cumulation may produce and export to the stem a substance or substances which behave as precursors of rubber. Such substances do not appear to be demonstrable in leaves of plants not engaged in active rubber accumulation. The active principle of this extract cannot be carbohydrate since the tissue is already supplied with a large amount of sucrose, a readily available carbohydrate. On the contrary, the influence of leaf extract can be duplicated by the pure chemical compounds acetate, acetone, and  $\beta$ -methylcrotonic acid. The structure of  $\beta$ -methylcrotonic acid in particular possesses the carbon skeleton found in isoprene, the fundamental unit of rubber, and  $\beta$ -methylcrotonic acid may thus be regarded as a possible precursor of rubber in the plant.

In the first paper in this series, it was shown that acetate, acetone, and  $\beta$ -methylcrotonic acid are able to support increased rubber synthesis in guayule seedlings. The results of the present investigations, in which an entirely different experimental technique has been used, confirm the results already reported.

#### SUMMARY

1. Isolated stem sections of *Parthenium argentatum* Gray, the guayule, were grown *in vitro* as material for testing the effectiveness of various materials in supporting the process of rubber synthesis.

2. Such stem sections make rapid and extensive callus growth when incubated on agar media containing mineral salts, sucrose, indoleacetic acid, and small amounts of other growth factors. During this growth, rubber is not accumulated above the concentration initially present.

3. Rubber accumulation in excised stem sections of guayule was promoted by addition to the medium of the extract of leaves from guayule plants which were themselves actively engaged in rubber formation. Similar extracts of leaves from guayule plants not engaged in active rubber accumulation were without any great effect in increasing rubber accumulation by excised stem sections.

4. Acetate, acetone, and  $\beta$ -methylcrotonic acid added to the medium used for cultivation of excised stem cuttings of guayule were effective in increasing rubber synthesis and accumulation. Other substances tested were ineffective.

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# Investigation of the Phosphorus-Containing Constituents of Centrifugally Prepared Fractions from Mouse Liver Cell Cytoplasm<sup>1</sup>

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## INTRODUCTION

In a previous report some over-all chemical analyses of various centrifugally prepared fractions from the cytoplasm of mouse liver cells were presented (1). In the present study a more detailed analysis of the phosphorus-containing compounds has been made, and the pentose nucleic acid of the various fractions studied in more detail.

## MATERIALS AND METHODS

The animals used, the preparation of the centrifugal fractions, and the analytical procedures employed were the same as described previously with the exception that in some of the experiments the livers were homogenized with a Potter-Elvehjem (2) homogenizer in place of the mortar and pestle grinding.

After these investigations were well under way, the paper of Hogeboom, Schneider, and Pallade (3) appeared describing the isolation of morphologically intact mitochondria from rat liver tissue employing 0.88 *M* sucrose as the suspending medium. When we applied this technique, we found it impossible with our equipment to complete the centrifugal separation of the *M* and *U* fractions as previously described. As these two components contained the greater part of the cytoplasmic pentose nucleic acid (PNA), it was decided, for the purpose of these studies, to continue with 0.85% sodium chloride as the diluting fluid. It should, therefore, be emphasized that in the separation from a saline suspension a poorer yield of mitochondria is obtained (3), and that the large granule fraction in which they appear also contains considerable material of microsome-like morphology as viewed in the electron microscope (4). The degree of this contamination is difficult to judge, but in our hands mitochondrial

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preparations sedimented from sucrose media have on two occasions had only about one-half the concentration of PNA generally found in large granule preparations obtained from saline suspensions.

Chemical separation of the phosphorus-containing compounds was carried out essentially as outlined by Schneider (5). In the earlier experiments the materials extracted as "lipide" in the Schneider procedure were subjected to a re-extraction with petroleum ether after they had evaporated to an oily residue. It was found that a rather large quantity of phosphorus that was originally soluble in alcohol and alcohol-ether was now insoluble in petroleum ether. However, when the lipide isolation was carried out by the more conventional method of extracting the fresh tissue preparation with 25 volumes of hot alcohol-ether (3:1), evaporating the extract to an oily residue and re-extracting this with petroleum ether, the values for lipide phosphorus thus obtained were found to agree within the limits of experimental error with those obtained by the Schneider procedure. Similar agreement between these two methods of isolation did not extend to the lipide nitrogen, which was always found to be much higher in the samples obtained by the Schneider procedure. It was found, however, that if the lipide fraction resulting from the Schneider fractionation was re-extracted with petroleum ether that portion of the lipide that remained petroleum ether soluble possessed the same N/P ratio as the entire lipide fraction isolated according to the other method.

These findings would indicate that in a Schneider separation, at least from mouse liver tissue, the phosphorus in the lipide fraction accurately reflects the lipide phosphorus as determined by a more conventional procedure. If an N/P ratio is to be determined, however, a subsequent petroleum ether extraction is necessary.

In order to characterize the nucleic acid of the liver cell cytoplasm more completely, quantitation of the phosphorus, pentose, nitrogen, and nitrogenous bases was carried out. Estimation of the nitrogenous bases was accomplished by means of ultraviolet

TABLE I  
*The Composition of Yeast Nucleic Acid and Its Ultraviolet  
Absorption at 260 mμ After Acid Hydrolysis*

Description of sample	Mg./ml. dialyzed YNA			Rib. P as % tot. P	N/P	N/rib.	E <sub>1%</sub> <sup>1cm.</sup> 260 mμ
	N	P	Ribose (× 2)				
Dialyzed	1.11	0.63	3.03	99.4	1.76	0.366	261
Dialyzed and acid- hydrolyzed	1.09	0.628	3.035	99.8	1.73	0.360	313
Dialyzed, acid-precipi- tated and acid- hydrolyzed	0.95	0.526	2.59	101.5	1.80	0.366	312
Dialyzed and acid- soluble	0.19	0.101	0.435	88.5	1.86	0.435	
Theoretical YNA				100.0	1.69	0.35	

absorption which was standardized with a sample of Boehringer yeast nucleic acid (YNA) kindly supplied to us by Dr. Albert Claude. To further purify this sample of YNA, it was dissolved in 5% sodium acetate, dialyzed against distilled water in the refrigerator and reprecipitated with 5% trichloroacetic acid (TCA). This reprecipitated YNA, as well as some of the dialyzed material, was hydrolyzed with 5% TCA at 90°C. for 15 min., and analyses carried out, the results of which are given in Table I. In the determination of the  $E_{1\text{cm}}^{1\text{cm}}$ , the concentration of YNA was calculated on the basis of the phosphorus determinations employing the theoretical per cent of phosphorus for YNA as 9.65. Since throughout this study the pentose nucleic acids were prepared for analysis by TCA precipitation and subsequent hydrolysis in 5% TCA, the value of 312 for the  $E_{1\text{cm}}^{1\text{cm}}$  at 260  $m\mu$  was employed when ultraviolet absorption data were quantitated.<sup>2</sup> The pH of the PNA solutions utilized for spectral analyses throughout this study varied from 1.8 to 2.7 and in this range the absorption at 260 and 280  $m\mu$  were found not to vary significantly.

## RESULTS

The fractions analyzed [for fractionation scheme see Ref. (1)] were: whole homogenate, *G*; cytoplasmic extract, *E*; the large granules that had been washed twice, *L*<sub>2</sub>; the microsomes washed once, *M*<sub>1</sub>; the fraction sedimented in 1 hr. at 95,000  $\times g$ , *U*; and the final supernate, *S*. After treating each of these fractions with 5% TCA, their acid-soluble constituents were analyzed and are recorded in Table II.

Despite precautions taken to keep the material cold throughout the prolonged separation procedures, it is evident that enzymatic activity has not been prevented completely since the final supernate contained slightly more acid-soluble nitrogen and appreciably more inorganic phosphate than did the cytoplasmic extract treated with TCA soon after the livers were removed. It is obvious that the factor of 2 which is based on the assumption that only 50% of the pentose gives a color with the Bial's reagent and which agrees well with phosphorus determinations on YNA (see Table I) is not valid for the acid-soluble pentose of the large granule fraction, since such calculations show more "pen-

<sup>2</sup> The increase in absorption at 260  $m\mu$  of PNA after hydrolysis with TCA has been studied in this laboratory and will be reported in detail at a later time. It can be said here, however, that such an increase is noted with both alkaline and acid hydrolysis, and that this increase in absorption is not obliterated by returning the hydrolyzed solutions to a neutral pH prior to spectral analysis. Although care has been exercised in all determinations reported here to correct for the TCA content of the hydrolysates by adding a similar amount of TCA to the blank solutions, it should be emphasized that TCA in the concentrations present in the solution used here for spectroscopy (0.5–.05%) has a very minor absorption in the range of 260 to 280  $m\mu$  and could not be responsible for the increased absorption noted.



tose" phosphorus than organic phosphorus. At least two factors may contribute to this discrepancy. Phosphatase activity might split phosphate from nucleotides so that the remaining organic phosphorus would be less than the calculated "pentose" phosphorus. The second and probably the major factor would be that appreciable amounts of the acid-soluble "pentose-phosphorus" compounds are independent nucleotides such as adenylic acid for which the factor of 2 is unwarranted. It is also appreciated that these two factors are affecting this particular determination in the other acid-soluble fractions such as *E*, but the discrepancies they produce are apparently masked by the presence of organic phosphate other than that associated with pentose.

TABLE II  
*Acid-Soluble Constituents of Cytoplasmic Fractions*

Fraction	No. of experiments	Mg. N/g <sup>a</sup>	Mg. P/g.	Mg. "Pentose"/g. (× 2)	Mg. inorg. P/g.	"Pentose" P as % org. P
<i>G</i>	8	1.79	0.805	2.81	0.267	108
<i>E</i>	16	1.65	0.725	2.12	0.315	109
<i>L<sub>2</sub></i>	12	0.098	0.047	0.189	0.017	127
<i>M<sub>1</sub></i>	11	0.085	0.026	0.07	—	—
<i>U</i>	4	0.039	0.009	0.025	—	—
<i>S</i>	6	1.71	0.71	0.75	0.43	54

<sup>a</sup> Throughout this paper the designation *mg./g.* (1) refers to the number of milligrams of a given constituent found in the cytoplasmic fraction isolated from 1 g. wet weight of liver tissue. All calculations are based on the assumption that the supernatant fluid decanted from a pellet contains the same concentration of nonsedimented constituents as did the original extract.

In general, therefore, it must be assumed that the estimation of acid-soluble pentose does no more than indicate the order of magnitude of pentose actually present. This is true not only because the probable presence of adenine nucleotides invalidates the factor of 2 but also because, especially in the case of the acid-soluble pentose of *S*, the presence of large amounts of such interfering substances as glucose (6) requires the use of a large and only approximate correction.

#### *Phospholipide Determination*

The results of the estimation of the phospholipide content of the various cytoplasmic fractions is given in Table III. During each cen-

TABLE III  
*Phospholipides of Cytoplasmic Fractions*

Fraction	Number of experiments	Mg P %	N/P (atom.c)
<i>G</i>	8	1.39	—
<i>E</i>	15	0.98	1.14
<i>L<sub>2</sub></i>	12	0.23	1.04
<i>M<sub>1</sub></i>	10	0.40	1.01
<i>U</i>	5	0.05	—
<i>S</i>	6	0.07	—

trifugation in the isolation procedure, varying amounts of fat rise to the surface of the tubes and are, in general, not included with the supernate when it is decanted. This probably accounts for the fact that the lipid phosphorus of *L*, *M*, *U*, and *S* adds up to only 75% of the lipid phosphorus of the whole cytoplasmic extract, with 68% of the phospholipide being in the particulates [see Ref. (7)]. From the data given in Table II of the first paper (1), it can be calculated that the particulates contain only 21% of the total lipid of the cytoplasmic extract (determined gravimetrically). It is then apparent that while the major portion of the cytoplasmic phospholipide is associated with the particulates, most of the neutral fat is "freely dispersed" in the cytoplasm.

#### *Nucleic Acid Determinations*

In the preceding investigation, the particulate fractions were found to contain 81% of the total pentose found in the four cytoplasmic fractions. From Table IV it may be seen that they contain 87.5% of the acid-precipitable nucleic acid of these fractions [see Ref. (8)]. Recovery experiments have indicated that the failure of the PNA of the fractions to add up to that of the whole extract is due largely to a loss of nucleic acid during washing of the large granule and microsome fractions; however, the possibility that some degradation of nucleic acid may occur during centrifugation can not be ruled out completely.

As may be seen from the column on pentose phosphorus as per cent of total phosphorus, it would appear that the factor of 2 used to calculate nucleic acid pentose from observed pentose is satisfactory for all fractions except the large granules. The nitrogen to phosphorus (N/P) ratios are all significantly above the theoretical ratio of 1.69, and probably indicate that a varying amount of protein hydrolysis has occurred

during heating with TCA. In the case of the *S* fraction where the ratio of protein to nucleic acid is very high, this nitrogen to phosphorus ratio has gone up to 5.72. The calculations given in the last three columns show in general a remarkably good correlation between PNA calculated from the determination of pentose, ultraviolet absorption, and phosphorus. Again, however, the large granule fraction differs significantly.

TABLE IV  
*Nucleic Acids of Cytoplasmic Fractions*

Fraction	No of experiments	Mg /g			Pentose P as % tot P	N/P	N/pentose	Mg PNA/g		
		N	P	Pent. (X 2)				From pentose	From U. V. abs	From P
<i>G</i>	8	2.40	0.996	3.93	81.5 <sup>a</sup>	2.41	—			
<i>E</i>	15	1.58	0.663	3.21	99.95	2.38	0.49	6.88	7.05	6.95
<i>L<sub>1</sub></i>	11	0.19	0.083	0.33	82.6	2.32	0.58	0.71	0.86	0.86
<i>M<sub>1</sub></i>	12	0.58	0.29	1.39	98.2	2.00	0.42	2.98	3.08	3.03
<i>U</i>	3	0.24	0.11	0.52	100.5	2.20	0.45	1.11	1.12	1.11
<i>S</i>	3	0.38	0.067	0.32	100.0	5.72	1.18	0.69	0.65	0.69
YNA theoretical					100.0	1.69	0.35			

<sup>a</sup> Approximately one-fourth of the phosphorus in this fraction is contributed by DNA. However, when the phosphorus contained in the PNA as estimated according to the HCl-orcinol reaction and that in the DNA as estimated by the diphenylamine reaction were added together they were found to equal 109% of that actually observed by direct phosphorus analysis. The reason for this discrepancy is as yet not clear.

Initial attempts to demonstrate desoxypentose nucleic acid (DNA) in any of the cytoplasmic fractions were without avail due to the relative insensitivity of the analytical methods. However, when the analysis of the nucleic acid fraction of the large granules consistently showed that "pentose" phosphorus accounted for only 70–90% of the total phosphorus, we again attempted to demonstrate DNA in this nucleic acid fraction. If any nuclei or nuclear fragments failed to sediment in the initial clearing run, they would automatically be concentrated in the large granule fraction and microscopic examination frequently demonstrates a few nuclear elements in this fraction following staining with methyl green. Consequently, two experiments were set up in which concentrated preparations of large-granule nucleic acid were prepared, and in these concentrates, it was possible to demonstrate DNA

by means of the Dische diphenylamine reaction (9). In one of these experiments the PNA phosphorus based on calculations from pentose determinations accounted for 80.5%, and the DNA phosphorus for 10.3% of the total nucleic acid phosphorus. In the other experiment, the PNA phosphorus accounted for 82.3% and the DNA phosphorus for 15.7% of the total nucleic acid phosphorus. It would appear, therefore, that the ratio of purines to pyrimidines in the pentose nucleic acids of all the cytoplasmic fractions is at least very close to 1 if one assumes that only their purine bound pentose reacts with the orcinol-HCl reagent (10).

The use of ultraviolet absorption, total phosphorus analyses, and pentose determinations as three independent analyses for these samples of PNA has proved most valuable, and should enable one to determine readily any significant change in the purine to pyrimidine ratio. The only discrepant finding in this comparison of liver cytoplasmic PNA with our purified sample of YNA was in the ultraviolet absorption spectrum. After hydrolysis with 5% TCA, the ratio of the absorption at 260  $m\mu$  to that at 280  $m\mu$  was 1.62 in the case of the YNA. In the samples from liver, the PNA had a 260 to 280  $m\mu$  ratio varying between 1.2 and 1.4.

These differences could possibly result from contamination of the liver PNA sample with aromatic amino acids as suggested by the elevated N/P ratios observed. They might, however, reflect some subtle difference between the YNA and the liver PNA samples studied. To investigate the latter possibility as well as to further characterize the liver PNA, further purification was undertaken.

The procedure suggested by Brues *et al.* (11) was adapted to this study. Since our material, with the exception of the  $L_2$  fraction, contained no DNA, separation of PNA from the major portion of the protein by hot 10% sodium chloride was carried out immediately. The PNA was then precipitated by the addition of 2 volumes of absolute alcohol and the precipitate dissolved in distilled water. For general chemical and spectroscopic analyses, the PNA was then precipitated with 5% TCA and hydrolyzed in 5% TCA for one-half hour at 80–5°C. Pentose estimations indicated that essentially quantitative yields were obtained in all steps of this procedure when mouse liver cytoplasm was used as the starting material.

Analyses of the PNA obtained in this manner revealed the same good correlation between the pentose, phosphorus, and spectral determina-

tions as was obtained when the Schneider procedure had been employed. However, the N/P ratio of the PNA obtained from all the cytoplasmic fractions by this latter procedure ranged from 1.8 to 1.9, quite in contrast to the results previously obtained. It is evident, therefore, that there could be little amino acid contamination in the samples prepared in this manner. When their absorption spectra were examined, they were found to differ very little from those previously described. The 260 to 280  $m\mu$  ratio for the PNA of  $M_1$  and the supernatant fluid,  $MS$ , remaining after removal of  $M_1$ , ranged from 1.27 to 1.36, significantly lower than that obtained from YNA of very similar chemical composition.

TABLE V

*Precipitation Characteristics of PNA Purified from Two Cytoplasmic Fractions*

Fraction	Number of experiments	Precipitating agent			
		5% TCA	0.4% $\text{CaCl}_2$	4% Ba acetate	Glacial acetic acid
		mg. P/g.	mg. P/g.	mg. P/g.	mg. P/g.
$M$	2	0.301	0.287	0.291	0.302
$MS^a$	2	0.116	0.046	0.058	0.008 <sup>b</sup>

<sup>a</sup> Supernatant fluid after two 90-min. centrifugations at  $23,000 \times g$ .

<sup>b</sup> Up to 57% of the TCA-precipitable PNA may be sedimented if centrifugation is carried out at  $2000 \times g$  instead of  $500 \times g$  at which all other centrifugations were done. In the case of the other precipitants, centrifugation in higher gravitational fields failed to sediment more PNA.

To further characterize the PNA of the liver cell cytoplasm, precipitation studies were carried out. Following alcohol precipitation in the above outlined purification procedure the samples were dissolved in distilled water, aliquots removed, and precipitation carried out by the addition of barium acetate (final concentration 4% adjusted to pH 6.8 to 7.2), calcium chloride (final concentration 0.4%), and glacial acetic acid (addition of 8 volumes). Precipitation was allowed to proceed overnight in the refrigerator and after washing, analysis was made of the precipitates. The results are given in Table V.

From these studies, it would appear that there are slight differences in precipitability of the PNA appearing in different locations in the cell cytoplasm. It is possible that the lack of complete precipitation of

the PNA in the supernatant fluid could reflect a partial degradation of the nucleic acid during centrifugation. This appears less likely in view of other experiments in which the cytoplasmic extract was treated immediately with hot 10% NaCl for purification of the PNA which was then compared with the PNA purified later from the supernatant fluid after two 90 min. centrifugations. In these experiments the barium-soluble PNA from the original extracts corresponded very closely in amount to that recovered from the final supernatant fluid.

### *Protein Residue Analyses*

After the cytoplasmic fractions have had their lipide and nucleic acid extracted by rather drastic means, there still remains a protein residue which accounts for most of the nitrogen of the fractions but which also contains a small amount of phosphorus. Initially it was felt that this remaining phosphorus might merely represent some nucleic acid that had not been completely removed. However, experiments with radioactive phosphorus have since shown that this protein phosphorus possesses a significantly higher specific activity than that of the nucleic acid phosphorus of the same centrifugal fraction. While this does not provide evidence of a complete chemical separation, it certainly indicates that there are two distinct phosphorus fractions in the nucleoprotein residue after removal of lipide and that at least the major part of the phosphorus in the protein fraction as obtained by this procedure probably is not nucleic acid phosphorus.

Table VI gives the composition of these protein residue fractions. The point of interest in Table VI seems to be that while the nitrogen

TABLE VI  
*Protein Residue Fractions of Cytoplasmic Particulates*

Fraction	Number of experiments	Mg. N/g.	Mg. P/g.	N P
<i>G</i>	8	23.0	0.139	165
<i>E</i>	15	17.3	0.086	202
<i>L<sub>2</sub></i>	11	3.24	0.032	102
<i>M<sub>1</sub></i>	11	2.73	0.024	114
<i>U</i>	5	0.80	0.0065	123
<i>S</i>	6	11.5	0.019	590

to phosphorus ratios for the three particulate fractions are remarkably similar, the ratio for the final supernate, *S*, is extremely high, indicating a much lower concentration of phosphoprotein in this fraction than in the particulates.

### DISCUSSION

The results of these more complete chemical characterizations of centrifugally prepared fractions of the cytoplasm of the mouse liver cell substantiate, in general, those results previously reported from this laboratory (1). While 72%<sup>3</sup> of the TCA-precipitable PNA of the cytoplasmic extract, *E*, was recovered in the particulate fractions, only 36% of the "protein" nitrogen appeared in these same fractions. It is evident, as suggested in the earlier report, that there is an appreciable amount (26% of that in the original extract) of TCA-precipitable nucleic acid remaining after the removal of the microsomal fraction, with 10% still remaining in the final supernatant fluid after 1 hr. centrifugation at  $95,000 \times g$ . Evidence is presented, however, indicating that the PNA remaining after the removal of the microsomes has somewhat different precipitation properties than does that appearing in the microsomes.

In the studies in which separate estimations of phosphorus, pentose, and nitrogenous bases of PNA were carried out, very good checks were obtained. In all instances, with the possibility of slight discrepancies in the large granule fraction, the results of the pentose analyses indicated that the nitrogenous base was, within rather narrow limits, made up equally of purines and pyrimidines, assuming that the orcinol-HCl procedure measures only the purine-bound pentose. The purified PNA obtained from the liver cell cytoplasm resembled our purified sample of YNA in all respects tested except that after hydrolysis with TCA the absorption at  $280 m\mu$  was relatively greater than was that of the YNA. The significance of this finding is not evident at present, but probably cannot be accounted for on the basis of contamination with aromatic amino acids.

Data concerning the distribution of TCA-soluble components reveal some interesting points. Although considerable effort was made to keep the samples well refrigerated throughout the prolonged separation procedure, both acid-soluble nitrogen and inorganic phosphorus were

<sup>3</sup> Eighty-seven per cent of the PNA recovered in the four centrifugal fractions appeared in the three particulate fractions.

more abundant in the final supernatant fluid than in the original extract. We have also observed a slight diminution in TCA-precipitable PNA and a 20% diminution in acid-soluble pentose upon allowing the final supernatant fluid to stand in an ice-water bath for 2 hr., suggesting some breakdown of PNA and a degradation or utilization of acid-soluble pentose at temperatures comparable to those maintained during centrifugation. In short, it seems evident that enzymatic activity is not completely abolished by temperatures in the range necessary to allow centrifugal separation of the cytoplasmic constituents. To date we have been unable to find an enzyme poison which would not also alter the centrifugal properties of the cytoplasmic constituents.

It would appear of great interest that although considerable time is consumed in the washing of the large-granule fraction an appreciable amount of acid-soluble pentose and phosphorus remains associated with this fraction. Observations in the electron microscope (4, 12, 13) strongly suggest that the granules morphologically identified as mitochondria, which are concentrated in this fraction, possess a well defined membrane. These chemical observations would seem to indicate that this membrane is able to retain an appreciable amount of the smaller nucleotides for the period of the centrifugation. The predominance of purine over pyrimidine nucleotides in this acid-soluble fraction further suggests the presence of adenine nucleotides within the mitochondria.

Electron microscopic observations in this laboratory have failed to demonstrate any such well defined membrane about the microsomal particles, and as prepared centrifugally, these particles contain relatively less TCA-soluble pentose. It would seem that the particles comprising the *M* and *U* fractions are essentially nucleoprotein-lipoprotein complexes. The most striking differences between them appears to be that the *U* particles contain a higher percentage of nucleic acid and a lower percentage of phospholipide [see Ref. (8)]. It should be realized, however, that the centrifugal separation of these submicroscopic particles probably represents only an arbitrary and imperfect cutting of a continuous spectrum. This point has been carefully investigated by Chantrenne (8).

It would appear that only a small beginning has as yet been made in this particular field of cellular biochemistry, and that as more data accumulate, the importance to the cell's economy of the intracellular



structure with localization of enzyme systems and substrates will become ever more evident.

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#### SUMMARY

The Schneider technic for the chemical fractionation of tissue extracts has been applied to the cytoplasmic fractions isolated from mouse liver tissue by differential centrifugation. These fractions have been characterized as to their acid-soluble, lipid, nucleic acid, and protein constituents. Marked differences have been observed between the three particulate fractions themselves, and also between these fractions and the nonsedimentable supernatant fluid.

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# Crystalline Lipoxidase. III. Amino Acid Composition <sup>1</sup>

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## INTRODUCTION

The oxidation of linoleic and related acids by soybean lipoxidase and similar enzymes from other sources has been the subject of considerable study. The available information on this enzyme system has been summarized in reviews by Süllman (1), Jezeski (2), and Bergström and Holman (3). Although attempts have been made to learn the mechanism of action of the purified enzyme (4) and the function of the enzyme *in vivo* (5), no explanation has been found for the enzyme's action. The study reported here was undertaken in order that the enzyme may be more fully characterized.

## EXPERIMENTAL

The crystalline lipoxidase used in these studies was the preparation described previously (6, 7). Two acid hydrolysates and one alkaline hydrolysate were prepared. One preparation of the enzyme, which had been stored as a precipitate in saturated ammonium sulfate, was exhaustively dialyzed against distilled water. The partially precipitated protein was discharged into a weighing bottle and dried in a vacuum desiccator over phosphoric anhydride to yield 21.1 mg. of dry substance, which was subjected to acid hydrolysis. A second preparation was treated in the same manner but was divided into two portions after drying. One portion, 26.3 mg., was hydrolyzed

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by autoclaving in 3.0 ml. 3.0 *N* HCl for 16 hr. The other portion, 18.1 mg., was hydrolyzed by autoclaving 16 hr. in 4.0 ml. of 4.0 *N* NaOH containing added cystine as stabilizing agent (8). Microbiological determinations were made on the two acid hydrolysates except in the case of tryptophan which was determined on the alkaline hydrolysate. In the latter case complete racemization during hydrolysis was assumed. The acid hydrolysates were freed of HCl by vacuum distillation. Small aliquot portions were removed and the amino acid composition determined by paper partition chromatography (9) at the Distillation Products Laboratories. Microbiological assays were performed on dilutions of these hydrolysates at A and M College on a semimicro scale (2 ml. total volume). The values obtained by microbiological analysis were in good agreement ( $\pm 5\%$ ) on repeated assays of both acid hydrolysates indicating similar purities for both enzyme preparations. The validity of the values was further checked by simultaneous analyses conducted on a sample of casein whose composition was known from repeated analysis made in this laboratory. Unfortunately, the limited amount of crystalline lipoxidase available did not permit additional hydrolysates to be made.

Two-dimensional chromatograms of aliquots equivalent to approximately 100  $\mu$ g. of lipoxidase were obtained. The sheets were developed first in water-saturated phenol and then in a 50-50 mixture of lutidine and collidine saturated with water. The locations of the amino acids were detected with ninhydrin. The general chromatographic procedure described by Ames and Risley (10) was used. Chromatograms were obtained in all three hydrolysates, the two acid hydrolysates giving identical results. Before development, the chromatograms were subjected to a number of different treatments including conversion to the acetate, the hydrochloride, and oxidation with hydrogen peroxide. These chromatographic techniques were used in order to extend the data obtained by microbiological assay to include those amino acids for which reliable microbiological methods were not available (alanine, cystine, serine, etc.) and for detection of any other substances that may be present.

## RESULTS AND CONCLUSIONS

The amounts of arginine, aspartic acid, glutamic acid, glycine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, proline, threonine, tyrosine, tryptophan and valine obtained by microbiological analysis are shown in Table I. These amino acids were also determined chromatographically and the quantitative data are included in Table I. Alanine, hydroxylysine, and serine were detected in the hydrolysates by paper chromatography. Cystine was not detected and it is concluded that this amino acid, if present, occurs in low concentrations. Hydroxylysine was indicated by the appearance of a spot at its reported location (16). No other amino acids tested yielded a similar spot, but since no authentic sample of hydroxylysine was available for comparison, its presence must be listed as provisional.

Certain discrepancies were observed in the microbiological and

TABLE I  
*Amino Acid Composition of Lipoxidase*

Substance	Paper chromatography <sup>a</sup> Moles/mole	Microbiological assay		
		Moles/mole	%	Reference
Alanine	60	—	—	—
Arginine	20	30	4.7	11
Aspartic acid	50	47	6.2	12
Cystine	0	—	—	—
Glutamic acid	110	73	10.4	13
Glycine	130	82	6.3	14
Histidine	15	22	3.6	15
Hydroxylysine	35 <sup>b</sup>	—	—	—
Isoleucine	200 (including phenylalanine)	63	8.1	17
Leucine	40	89	11.4	17
Lysine	10	54	7.8	19
Methionine	—	13	1.8	20
Niacin	Included with leucines	0	0	21
Phenylalanine	0	30	4.9	18
Proline	80	46	5.1	14
Serine	40	—	—	—
Threonine	0	53	8.9	15
Thyroxine	0	—	—	—
Tryptophan	40	4	0.41	20
Tyrosine	50	35	6.2	20
Valine	110 (not included in total)	65	7.8	17
Unknown No. 1				
Total amino acid residues		881		
Sum of moles × mol. wt.		111,984		
— 881 H <sub>2</sub> O		15,858		

Molecular weight accounted for 96,162

<sup>a</sup> These figures are only estimations obtained by spectrophotometric assays of the intensities of the ninhydrin colors. Appreciation is expressed to Dr. J. Thompson of the University of Rochester who assayed a sample by quantitative paper chromatography and obtained comparable results.

chromatographic data. Proline was not detected by chromatography, but 5.1% proline was found by microbiological analysis. This discrepancy is as yet unexplained. Although tryptophan was not shown to be present by chromatographic analysis, the amount of tryptophan deter-

mined by microbiological analysis was too low to be detected on the chromatogram.

Lipoxidase was found to contain 1.6% methionine by microbiological assay, but at first it was not detected by paper chromatogram. It was suspected that lanthionine (suggested provisionally by chromatography) accounted for part of the methionine activity detected microbiologically.

However, lanthionine obtained from Bios Laboratories, mesolanthionine supplied by Dr. D. Breese Jones, and L-lanthionine obtained from Dr. V. du Vigneaud showed no activity for *Leuconostoc mesenteroides* P-60 when substituted for methionine. When the amount of lipoxidase hydrolysate used on the chromatogram was doubled, a faint spot corresponding to methionine sulfoxide was observed. The spot on the paper chromatograms in the area assigned to lanthionine (16) migrated somewhat less rapidly than glycine in phenol and about one-half the speed of glycine in lutidine-collidine. The location of the spot could not be altered by pretreating the hydrolysate with hydrogen peroxide, ammonia, or acid. Since chromatography provisionally indicated that this substance was lanthionine, authentic samples of lanthionine were tested. These did not appear at the location on the chromatogram corresponding to the substance thought to be lanthionine, thus indicating that this acid is not present in lipoxidase. From the location on the chromatogram, this unknown substance is possibly a sulfur-containing amino acid, but its identity must remain in doubt until the amino acids in this area have been investigated further.

These comparisons obtained by the completely independent microbiological and chromatographic methods are of considerable interest. The high sensitivity of the methods permits a rather complete amino acid analysis of small amounts of protein. As further improvements in the methods become available, their application to these and other problems will be greatly increased.

A summary of the data obtained by microbiological assay and by estimation from paper chromatography is compiled in Table I. The approximate number of residues of the amino acids is given, and wherever available, the value obtained by microbiological assay is used in the calculations. The summation of the molecular weights of the amino acids measured or estimated accounts for approximately 94% of the lipoxidase molecule whose molecular weight is 102,400 (7),

correction being made for loss of water in formation of peptide linkages. The number of amino acid molecule residues accounted for is 881, the molecule containing approximately 938 amino acid residues.

The amino acids which account for nearly all the light absorption of proteins at 2800 Å are tyrosine and tryptophan. Lipoxidase has been found to have a light absorption at 2800 Å of  $E_{1\text{cm}}^{1\%} = 17.8$  (7), a value slightly higher than that of an average protein. It was thought that this was due to a high content of tyrosine and tryptophan, but the contribution of these amino acids to the extinction at this wave length amounts to only  $E_{1\text{cm}}^{1\%} = 5.7$ . Apparently then the enzyme must contain some group or residue with a relatively high ultraviolet absorption.

Comparison of these results with the recent analyses of aldolase and D-glyceraldehyde phosphate dehydrogenase (22) indicates certain differences in composition between lipoxidase and these enzymes. The amount of tryptophan and the sulfur-containing amino acids are low in lipoxidase, cystine possibly being absent. Of the amino acids measured, leucine and glutamic acid are present in the highest amounts.

The nature of the active center in lipoxidase is still not known. The absence of mercapto groups was indicated by the inefficacy of enzyme inhibitors known to bind —SH groups (4), and by a negative nitroprusside test. Heavy metals have been eliminated as active centers because heavy-metal inhibitors do not inhibit the enzyme and because iron was not found in the enzyme in sufficient quantity for an atom per mole (7). The ability of the repeatedly-dialyzed enzyme to function in a simple system precludes the existence of a necessary coenzyme or dissociable prosthetic group. The character of the absorption spectrum of the pure enzyme (7) precludes the presence of a tightly bound prosthetic group of a common resonating type. All indications are that the enzyme is a simple globulin. However, the amino acid composition indicates the possibility of the presence of some amino acids or other substances which may account for a large portion of the ultraviolet absorption of the enzyme.

#### SUMMARY

The amino acid composition of crystalline lipoxidase has been investigated using both two-dimensional paper chromatography and microbiological methods. The presence of the following amino acids has been indicated: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxylysine, isoleucine, leucine, lysine, methi-

onine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. An unknown constituent was also indicated by paper chromatography. Cystine, thyroxine, and niacin were not detected. Lanthionine has been found unable to substitute for methionine as a requirement for *Leuconostoc mesenteroides* P-60.

The ultraviolet absorption of lipoxidase at 2800 Å cannot be accounted for by the tyrosine and tryptophan content of the enzyme, indicating that some substance having a relatively high ultraviolet absorption must be present. Ninety-four per cent of the composition of the molecule has been accounted for. The enzyme probably contains about 938 amino acid residues.

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# Comparative Studies on Nitrogen Excretion<sup>1</sup>

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Sprinson and Rittenberg (1) have recently shown that N<sup>15</sup>-labeled glycine may be used to indicate the rate of synthesis of protein in man and animals.

In utilizing a similar procedure to study certain abnormal states of protein metabolism, we have had occasion to compare control experiments on normal individuals using both tagged glycine and whole dried yeast labeled with N<sup>15</sup>. The glycine used contained 4.68% excess N<sup>15</sup>. Isotopic yeast was prepared by growing bakers' yeast in a medium of glucose and inorganic salts, the nitrogen constituent of which contained N<sup>15</sup>. The dried yeast obtained in this manner gave on analysis a protein content of 49.6% (7.95% N  $\times$  6.25), and an N<sup>15</sup> concentration of 6.51% excess. Di Carlo *et al.* (2) have shown that the various nitrogenous constituents of yeast contain relatively the same concentrations of N<sup>15</sup> when grown under similar conditions.

The three subjects used throughout these experiments were normal adult males weighing 98, 74, and 80 kg. Dietary protein was supplied at a level of 1 g. /kg. body weight /day on a normal diet. Calories totaled 2700, 2400, and 2500, respectively. Glycine was fed at a level of 125 mg. /kg. body weight, and yeast protein as 13.5% of the dietary N. In all metabolic periods, unmarked glycine or yeast was supplied daily with breakfast. Three to 4 days were allowed for attaining nitrogen equilibrium on the diet. The isotope-containing constituent of the diet was then substituted for one breakfast feeding and the N<sup>15</sup> and total

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nitrogen excretion followed for a period of 3 days. Urine samples were collected for four 6-hr. periods the first day, two 12-hr. periods the second, and one 24-hr. period the third.

Total nitrogen determinations were made by the usual Kjeldahl procedure. Samples were prepared as  $(\text{NH}_4)_2\text{SO}_4$  for mass spectrometer analysis. Nitrogen was obtained from the  $(\text{NH}_4)_2\text{SO}_4$  by the procedure of Rittenberg *et al.* (3) using NaOBr.

In Table I are given the analytical data from a typical series of experiments.

TABLE I  
*Analytical Data of a Typical Experiment*  
Subject: A. W.  
Daily Caloric Intake: 2700 cal.  
Daily Nitrogen Intake: 16.1 g.

N <sup>15</sup> source	Total N <sup>15</sup> fed	Urine values							
		6 hr.		12 hr.		18 hr.		24 hr.	
		Total N	N <sup>15</sup>	Total N	N <sup>15</sup>	Total N	N <sup>15</sup>	Total N	N <sup>15</sup>
Glycine	mg. 104	g. 4.37	0.240	g. 3.67	0.230	g. 3.25	0.165	g. 3.55	0.140
Glycine	104	3.82	0.270	3.41	0.230	2.77	0.185	3.23	0.150
Yeast	140	3.07	0.233	3.93	0.240	3.94	0.195	2.17	0.155

<sup>a</sup> Expressed as per cent excess.

Table II shows the results of the isotope analysis of both sets of experiments. From the data presented it is obvious that yeast nitrogen is excreted more slowly than that of glycine. That this difference is not due to an imbalance of amino acids, *i.e.* an excess of glycine, would appear from the satisfactory agreement of our data with that reported by Sprinson and Rittenberg. On this basis, an added glycine intake varying from 600 mg. to 12 g. has little or no effect on the rate of N<sup>15</sup> excretion.

The discrepancy may be explained in part upon the slower rate of absorption of yeast nitrogen, due to the necessity of prior digestion. Figure 1 shows the results of one experiment with yeast in which the urine N<sup>15</sup> concentration was measured at hourly intervals during the

TABLE II  
Comparative  $N^{15}$  Excretion Values

Subject	N source	Periods in hours						
		0-6	6-12	12-18	18-24	24-36	36-48	48-72
A. W.	Glycine	9.9	17.5	22.4	27.1	34.3	38.5	40.8
A. W.	Glycine	10.1	18.2	23.4	28.2	33.7	37.9	41.3
A. W.	Yeast	5.1	11.8	17.3	19.7	25.6	28.8	32.7
R. H.	Glycine	9.0	17.2	23.0	27.0	34.9	40.5	45.8
R. H.	Yeast	4.5	10.7	15.2	18.4	22.7	25.6	30.5
R. H.	Yeast	3.7	9.2	13.7	16.6	22.1	25.8	30.6
K. C.	Glycine	9.9	18.6		27.4		39.2	44.2
K. C.	Yeast	2.9	8.0	11.1	14.0	18.1	21.1	25.0

<sup>1</sup> Values expressed as additive percentages of the  $N^{15}$  ingested.

first period. A relatively constant rate of elimination appears to be reached between the 2nd and 3rd hr. after breakfast, continuing for at least 12 hr. In addition, each individual amino acid may have its own rate of turnover in the nitrogen pool. Our results would therefore rep-

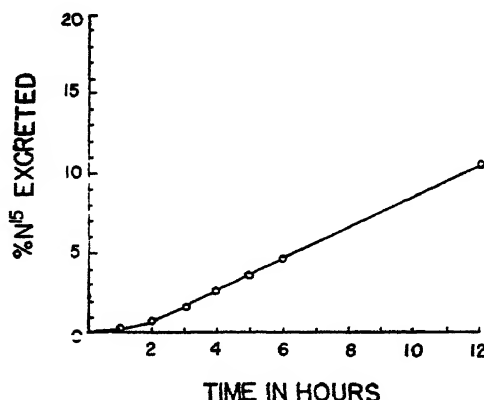


FIG. 1. Excretion of yeast nitrogen.

resent only an average value dependent upon the concentrations and rates of the individual compounds concerned.

Further work obviously is needed to substantiate and explain the differences observed.

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# Colorimetric Determination of the Pyrimidine Content of the Desoxyribonucleic Acids of Thymus and Fish Sperm<sup>1</sup>

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## INTRODUCTION

Renewed interest in the field of nucleic acids has led to the development of new methods for the analytical study of these complex substances. A recent example of the application of such methods is the work of Chargaff and co-workers (1), who have studied the distribution of the purines and pyrimidines in the hydrolysates of desoxypentose nucleic acids derived from calf thymus and beef spleen. These investigators employed paper chromatography for the separation of the nitrogenous constituents present in the hydrolysates, together with spectrophotometric methods for the estimation of the individual purines and pyrimidines.

The present study deals with the colorimetric determination of both the cytosine and thymine content of the desoxypentose nucleic acids of fish sperm and thymus. Two different colorimetric methods have been employed. A colorimetric test for the estimation of cytosine has been developed in this laboratory (2) which can be applied to hydrolysates of nucleic acids. This test is not given by thymine. The second method is based on a color test developed by Hunter (3) for distinguishing thymine from uracil or cytosine by employing the diazo-reagent of Koeßler and Hanke (4). We have found that Hunter's test when used under the conditions described below can be applied to the quantitative estimation of thymine in hydrolysates of desoxypentose nucleic acid. While this work was in progress, Woodhouse (5) reported a method for the colorimetric estimation of thymine which is also based on Hunter's test. However, this investigator isolated thymine from the hydrolysate of nucleic acid by a lengthy procedure before applying the test.

<sup>1</sup> This investigation was aided in part by a grant from the Damon Runyon Memorial Fund.

## EXPERIMENTAL

*Materials*

The thymine, cytosine, guanylic, and adenylic acids were analytically pure.

*Desoxyribonucleic Acid (DNA).* Three samples of DNA were used. Sample 1 was a sodium salt of fish sperm DNA.<sup>2</sup> Sample 2 was a sodium salt of calf thymus DNA.<sup>3</sup> Both were purified according to the method of Chargaff *et al.* (1). Sample 3 was isolated by us from calf thymus according to the procedure of Hammarsten (6). All three samples were found to be free from protein and pentose.<sup>4</sup>

*Procedure and Results*

*Qualitative Estimation of Thymine in Pure Solution.* The only modification of Hunter's test which we have made has been the time of coupling. We allowed the coupling reaction to proceed for exactly 10 min. as compared with 5 min. in Hunter's test. This extension of the time of the reaction gives a greater amount of color production.

The procedure is carried out at room temperature. After allowing the color to develop for 10 min., the color intensity is measured in a Klett-Summerson colorimeter using a No. 52 filter.

*Determination of Cytosine.* The procedure for the colorimetric estimation of cytosine described in a previous paper (2) was followed exactly.

*Estimation of Thymine in Hydrolysates of DNA.* On hydrolyzing DNA to yield free cytosine and thymine, there are also released at the same time the purine bases and the degradation product of desoxyribose, levulinic acid. Hunter's reaction cannot be directly applied to the hydrolysate because of the presence of the purines and of levulinic acid which interfere with the test. We have found that the purines can be quantitatively removed by treatment of the mixture with palladous chloride (7). The levulinic acid is removed by extraction with ether. After the removal of these interfering substances, the Hunter reaction can be applied directly to hydrolysates of nucleic acid provided two blanks are used. One blank consists of 1 ml. of distilled water to which all the reagents have been added. This blank is used for obtaining the reading of the standard. A second blank must be employed because of the formation of a distinctive yellow tint in the nucleic acid hydrolysate

\* Supplied by Krishell Laboratories, Inc., Portland, Oregon.

<sup>2</sup> Supplied by Schwarz Laboratories, Inc., New York.

<sup>4</sup> After drying *in vacuo*, Sample 1 contained 15.2% N (Dumas) and 8.9% P (Pregl-Lieb); Sample 2, 13.6% N and 8.0% P; and Sample 3, 13.5% N and 7.9% P.

during the coupling reaction and which does not occur in the case of the thymine standard. The second blank consists of an aliquot of the nucleic acid hydrolysate which has been run through the entire test except for the last step, namely, the addition of the hydroxylamine hydrochloride for the development of the red color. This blank is used for obtaining the readings due to the thymine content of a similar aliquot of the nucleic acid hydrolysate.

*Determination of Cytosine in Hydrolysates of D.N.A.* The colorimetric method for the determination of cytosine (2) can be applied directly to the nucleic acid hydrolysate after the removal of the purines.

*Determination of the Pyrimidine Content of D.N.A.* The nucleic acid may be hydrolyzed by either of two methods. The first (Method I), in which the hydrolysis is carried out with concentrated formic acid, yields both the cytosine and thymine almost completely intact. Analyses for both thymine and cytosine were carried out with these hydrolysates. The second (Method II), which uses 24% sulfuric acid to hydrolyze the nucleic acid, cannot be used to determine the cytosine, since part of it is deaminized. Therefore, this procedure was used only for the determination of thymine in the hydrolysates.

*Method I.* The nucleic acid (50–75 mg.) was placed in a small Pyrex bomb tube and treated with 0.5–1.0 ml. of concentrated formic acid (1). The tube was sealed and heated for 2 hr. at 175°. The resulting dark-brown solution, which was left in the tube, was evaporated to dryness *in vacuo* over calcium chloride and potassium hydroxide. The residue was extracted with anhydrous ether to remove the last traces of formic acid, centrifuged, and the ether poured off. The extracted residue was dissolved in 0.1 N hydrochloric acid and quantitatively transferred to a small beaker with small quantities of water. The pH was adjusted to 1.4, and 12 drops of 2% palladous chloride was added. The mixture was heated to boiling, cooled, and filtered. The flocculent precipitate was washed with cold water, and the washings added to the filtrate. The filtrate was then brought to the same pH as that of the thymine standard (pH 5.5–6.0), and the volume adjusted to exactly 50 ml. Any precipitate forming at this point is filtered off. The cytosine is determined colorimetrically in 1-ml. aliquots of this solution. For the estimation of thymine, 5 ml. of the solution was pipetted into a centrifuge tube and evaporated to dryness. The residue is extracted with 5 ml. of anhydrous ether, the mixture centrifuged and the ether poured off. This step is necessary to insure complete removal of the levulinic acid. The extracted residue is air-dried, treated with 5 ml. of distilled water, and allowed to stand overnight. Any residue remaining undissolved is discarded. One-ml. aliquots of this solution are then analyzed for thymine by means of Hunter's test.

*Method II.* The nucleic acid (50–75 mg.) was placed in a small Pyrex bomb tube. One ml. of 24% sulfuric acid was added, and the sealed tube heated at 125° for 25 hr. The resulting dark-brown solution was transferred quantitatively to a small beaker

and brought to pH 1.4 with sodium hydroxide. The precipitation with palladium chloride and the subsequent steps were carried out as above.

*Results of Analyses of Nucleic Acid Specimens for Cytosine and Thymine.* The results obtained in determining the pyrimidine content of sperm and thymus DNA are summarized in Table I. The data obtained in this study are in fair agreement with those reported by Chargaff and co-workers, and by Woodhouse. Chargaff *et al.* found an aver-

TABLE I  
*Pyrimidine Content of Three Different Samples of Desoxyribonucleic Acid<sup>a</sup>*

Sample	Method I		Method II
	Thymine	Cytosine	Thymine
1	%	%	%
	8.6	5.7	8.0
	7.9	5.3	8.2
	8.0	5.6	8.8
	8.2	5.6	8.9
			8.8
2			9.0
			9.0
3	8.4	4.7	
	8.6	4.7	
	8.8	4.6	
3	8.3	4.6	
	8.4	4.7	
	8.7	4.9	

<sup>a</sup> Each value represents the average of five separate analyses on the same hydrolysate.

age of 5.8% cytosine and 7.9% thymine in calf thymus DNA, and 5.1% cytosine and 8.5% thymine in beef spleen DNA. Woodhouse reported 9.0% thymine in calf thymus DNA.

*Determination of Thymine and Cytosine in the Presence of Guanylic and Adenylic Acids.*<sup>5</sup> Known quantities of thymine and cytosine were placed in a bomb tube, together with amounts of guanylic and adenylic

<sup>5</sup> The guanylic and adenylic acids used contained ribose, since the desoxyribose nucleotides were not available.

TABLE II  
*Determination of Thymine and Cytosine in the Presence  
of Guanylic and Adenylic Acids*

	Amount in mixture		Amount recovered	
	Cytosine	Thymine	Cytosine	Thymine
Method I	mg	mg	mg	mg
	4.25	2.75	4.20	2.50
	4.24	2.79	4.28	2.51
Method II	—	2.78	—	2.70
	—	2.69	—	2.63

\* Each value represents the average of five separate analyses on the same hydrolysate.

acids corresponding to the quantities of guanine and adenine believed present in 50 mg. of DNA. The hydrolyses were carried out according to Methods I and II, and the hydrolysates were analyzed for cytosine and thymine as described above. Table II shows the data obtained.

### SUMMARY

Colorimetric methods for the estimation of thymine and cytosine in the presence of each other are described. These methods have been applied to the determination of the thymine and cytosine content of fish sperm and thymus deoxyribonucleic acid.

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# Biotin in the Assimilation of Heavy Carbon in Oxalacetate

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## INTRODUCTION

Early experiments on heterotrophic assimilation of carbon dioxide indicated that biotin plays the role of coenzyme in the Wood-Werkman reaction. Specifically, the quantity of succinic acid formed during the anaerobic dissimilation of pyruvic acid by a juice of *Escherichia coli* was a function of the biotin present. This result is to be expected if biotin functions in the  $\beta$ -carboxylation of pyruvate. Since this initial work did not yield conclusive results because of technical difficulties, the problem now has been attacked differently. It is felt that the present results are conclusive proof of the role of biotin in  $\text{CO}_2$  assimilation. Considerable work has appeared on the function of biotin, particularly from the laboratories of Shive, Lardy, and Lichstein (1-7), which supports the role of biotin in the enzymatic fixation of  $\text{CO}_2$  in oxalacetate.

The present attack differs from any reported in that heavy carbon has been employed as a tracer and avidin as an inactivator of biotin. In general, the method employs heavy carbon in the reaction involving the enzymatic decarboxylation of oxalacetate first reported by Kramnitz and Werkman (8). Avidin is added to inactivate the biotin to determine its effect on  $\text{CO}_2$  fixation, as shown by changes in  $\text{C}^{13}$  fixed in oxalacetate.

## METHODS

*Micrococcus lysodeikticus*, used as the source of enzyme, was grown on dextrose yeast agar in Roux flasks at  $30^\circ\text{C}$ . for 72 hr. Both acetone-treated and lyophilized cells were used. In most of the experiments the cells were lysed by the addition of lysozyme.

In early experiments egg white was used rather than avidin to inactivate the biotin. However, its use introduces complications due to its composition and makes the evidence questionable, so avidin was later used.

The amount of egg white or avidin necessary to inactivate the biotin in the cells under experimental conditions was first determined. This involved a slight modifica-

tion of the biotin assay method of Roberts and Snell (9). Lysed cells were added to the basal medium to serve as the source of biotin, and various concentrations of egg white or avidin were added to the assay tubes. These were inoculated with *Lactobacillus arabinosus* and growth determined by titration of the lactic acid formed after 72 hr. or by measurement of turbidity with a Klett-Summerson photoelectric colorimeter after 16 hr. The two methods gave results which checked closely. In all except the early work, the turbidimetric method was used because of the shorter time interval involved. Table I shows results of typical inactivation assays.

Attempts to sterilize solutions of avidin before adding it to the assay medium by passing it through a Seitz filter resulted in inactivation due to its failure to pass through the filter.

TABLE I

*Inactivation of Biotin by Egg White and Avidin*  
Solution of lysed *Micrococcus lysodeikticus* as source of biotin

Cells	Egg white	Avidin	Growth response	
			Colorimeter reading <sup>a</sup>	0.1 N Lactic acid
mg./cc.	0.2 mg./cc.	μg.		ml.
1.0	0.0		27.0	15.39
1.0	0.1		22.0	14.48
1.0	0.5		16.0	11.41
1.0	1.0		9.0	8.51
1.0	2.0		6.0	4.69
1.0	3.0		6.0	4.40
1.0		0.0	37.0	
1.0		0.1	33.0	
1.0		0.5	19.5	
1.0		1.0	11.5	
1.0		10.0	11.0	
1.0		50.0	10.5	
1.0		100.0	9.0	
1.0		200.0	10.0	

<sup>a</sup> Klett-Summerson.

Exchange reactions were conducted in 125-ml. two-arm Erlenmeyer flasks attached to Barcroft-Warburg manometers. Appropriate additions were made to each flask and the reaction allowed to go approximately half way to completion when the oxalacetate was immediately degraded and C<sup>13</sup> content determined on the mass spectrometer.

Crystalline avidin was obtained by ammonium sulfate extraction of egg white (11, 12). Cells were lysed by a 10-min. incubation with 1.5% lysozyme before adding to the reaction mixture.

## EXPERIMENTAL

Any effect of biotin on  $\text{CO}_2$ -fixation was determined by observing the action of lysed cells in the presence of synthetic oxalacetate and  $\text{NaHC}^{13}\text{O}_3$ . Egg white or avidin was added to inactivate the biotin in the cells. A typical experiment was set up as follows: Each cup contained 0.05 *M* oxalacetate, 0.025 *M* phosphate buffer, pH 6.6, 0.053 *M*  $\text{NaHC}^{13}\text{O}_3$  (5.13 atom per cent excess) and 300 mg. of lysed cells. Cup 1 served as a normal control, Cup 2 contained added avidin (or egg white), and Cup 3 avidin plus biotin to compensate for that inactivated by the avidin. The cups were equilibrated at 30.4°C. for 10 min. before adding oxalacetate and bicarbonate, and the reaction was allowed to proceed 20 min. (approximately half completion). The oxalacetate remaining was immediately degraded by the acid and heat method (10), which involves aeration at pH 4.0 to remove residual  $\text{CO}_2$ ; this was followed by a bicarbonate flushing and boiling at pH 4.0 to free the  $\text{CO}_2$  from the methylene carboxyl group, and the  $\text{C}^{13}$  was then determined.

Early experiments were conducted in which we observed the effect of biotin on  $\text{CO}_2$  fixation in "physiological oxalacetate" resulting from fumarate oxidation. Fumarate was added to the exchange vessels and the reaction studied in the presence of acetone-treated cells and  $\text{NaHC}^{13}\text{O}_3$ .

The results (Table II) obtained show a marked decrease in  $\text{C}^{13}\text{O}_2$  fixed when a biotin inactivator is added, whereas the addition of biotin results in a reversion to the normal exchange. In the case of lysed cells

TABLE II  
*Effect of Biotin on Heavy-Carbon Exchange During  
Enzymatic Decarboxylation of Oxalacetate*  
Cells lysed by treatment with crystalline lysozyme.

Normal exchange	Egg white added	Egg white + equivalent biotin	Avidin added	Avidin + equivalent biotin	Avidin + excess biotin
0.14 <sup>a</sup>	0.02	0.12			
0.17			0.03	0.15	0.16
0.42 <sup>b</sup>	0.11	0.22			

<sup>a</sup> Values are given as excess atom per cent  $\text{C}^{13}$ .

<sup>b</sup> Decarboxylation of "physiological oxalacetate" (from fumarate).

acting on synthetic oxalacetate the decrease is 90–100% when egg white or avidin is added, whereas the addition of biotin restores the value practically to that of a normal exchange. When acetone-treated cells and “physiological oxalacetate” were employed the decrease in  $C^{13}O_2$  fixed is definite but not as complete, and the addition of biotin restores approximately one half.

### DISCUSSION

Since avidin prevents the fixation of  $CO_2$  by *Micrococcus lysodeikticus* and this effect is removed by the addition of biotin, the evidence is conclusive that biotin functions as a catalyst in the Wood-Werkman reaction. The fixation of  $CO_2$  is not completely prevented in the presence of avidin; this is to be expected, first because not every molecule of biotin would be bound by the added avidin and secondly, the experimental error of the mass spectrometer will account for part of the difference. To avoid problems posed by permeability considerations, lysed cells have been used. Since the results with acetone-treated cells did not show as complete reduction of  $CO_2$ -fixation, it is to be assumed that permeability effects are involved.

Although biotin may be active in other reactions, it is likely that its role in  $CO_2$ -fixation in oxalacetate is one of the more important. The exact mechanism is not clear and a solution must await further research.

### SUMMARY

In exchange reactions involving oxalacetate and  $NaHC^{13}O_3$ , fixation of carbon dioxide in oxalacetate is prevented when avidin is added to inactivate the biotin. The addition of adequate biotin results in a return to normal fixation. The evidence appears conclusive that biotin functions as a coenzyme in the fixation of carbon dioxide.

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# Extraction Studies of Sheep Pituitary Gonadotropic and Lactogenic Hormones in Alcoholic Acetate Buffers

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## INTRODUCTION

Since the demonstration by Smith and Engle (1) and Aschheim and Zondek (2) of the gonadotropic effects of implantations of anterior lobe tissue, numerous methods of extracting the gonadotropic activity from anterior pituitary tissue have been used. Alkaline extracts, acid extracts, water extracts, saline extracts, alcoholic extracts, and acetone extracts have been used to obtain the concentrated gonadotropin from the anterior pituitary tissue. Weisner and Marshall (3) came to the conclusion that the gonadotropic hormone consisted of two separate hormones. Their conclusion was based upon the observation of Smith (4) who observed that pituitary implants produced chiefly a follicular response with little luteinization. Also, Evans and Simpson (5) reported that alkaline extracts were more strongly luteinizing than were acid extracts. Fevold, Hisaw, and Leonard (6) claimed to have fractionated pituitary gonadotropic extracts into follicle stimulating hormone (FSH) and luteinizing hormone (LH). Later this finding was confirmed by Van Dyke and Wallen-Lawrence (7), and Evans *et al.* (8). These results were based upon the rather laborious bioassay method of which there are various modifications (9, 10), none of which is very accurate.

It was observed (10) that human and horse pituitaries are rich in FSH, but poor in LH. Cattle pituitaries are extremely weak in gonadotropin. In most of the work, either sheep or hog pituitaries have been used, because they were more plentiful at the time and contain appreciable amounts of gonadotropin. Evans *et al.* (8, 11) utilized the

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greater solubility of FSH over LH in  $(\text{NH}_4)_2\text{SO}_4$  solutions to separate the FSH and LH fractions. Fevold's method (12, 13, 14) depended on greater solubility of FSH in certain concentrations of  $(\text{NH}_4)_2\text{SO}_4$  or acetone. LH shows minimal solubility at pH 4.2. Wallen-Lawrence (15) separated LH from FSH by precipitation with alcohol at  $-6^\circ\text{C}$ . The precipitate at 40% alcohol was predominantly LH; the precipitate at 55% alcohol was predominantly FSH. Greep *et al.* (16) found hog LH extremely insoluble in 0.25 *M* acetate buffer at pH 4.4 and  $20.5^\circ\text{C}$   $\text{Na}_2\text{SO}_4$ . FSH was very soluble under these conditions. Recently Li (21) announced the separation of pure FSH.

Inasmuch as two independent groups (16, 17) using pituitary tissue from two different species have claimed to have isolated the pure LH hormone, homogeneous with respect to electrophoresis, ultracentrifuge, and solubility criteria, a brief review will be given of this work.

The method of Van Dyke *et al.* (16, 18, 19) for the extraction and purification of LH hormone from hog pituitaries consisted of a rather elaborate utilization of differential solubility in  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{Na}_2\text{SO}_4$  solutions with alteration in pH at various stages. This method gave a homogeneous protein-like material possessing LH activity. The method of Evans *et al.* (17), on the other hand, uses sheep pituitaries. This method employs alcohol, acetone,  $(\text{NH}_4)_2\text{SO}_4$ , pH 4.5, and final purification by precipitation with trichloroacetic acid. This method gave a homogeneous protein-like material possessing LH activity.

In all the above methods, the pituitary glands were extracted for the gonadotropic hormone alone. For some time, especially for economic reasons, there has been a need for a method whereby more than one of the pituitary hormones could be extracted from one and the same gland. Bonsnes and White (20) attempted to do this by fractionating a 2% NaCl extract of beef anterior pituitary tissue by adjusting pH and by the addition of acetone in step-wise fashion. Some fractionation was obtained, but there was considerable overlapping of activities in the various fractions. Fevold *et al.* (14), using whole sheep pituitaries, attempted a fractionation using  $(\text{NH}_4)_2\text{SO}_4$  as the precipitating agent and dilute aqueous ammonia as the extracting medium. He was able to separate five of the anterior pituitary hormones into five different protein fractions.

In the light of the work mentioned above and of the development of methods (22) by which plasma and serum proteins could be fractionated utilizing ethanol precipitation of the proteins at low temperatures,

work was undertaken to fractionate whole sheep pituitaries. The purpose was to get as many of the pituitary hormones from a single gland in as high potency as possible. Effort was concentrated upon the gonadotropin.

### METHODS

In order to determine the potency of the preparations, it was necessary to rely on a biological assay. For this assay, 21-22-day-old normal female Sprague and Dawley rats weighing between 35 and 50 g. each were used. The unit used was the Collip unit which is one-tenth of the daily dose, which, when administered 3 times a day for 3 days, will produce ovaries weighing 35 mg. All materials were assayed against a standard; this consisted of an arbitrary, potent, lyophilized gonadotropin powder prepared at the laboratory, stored over  $P_2O_5$  in a vacuum desiccator, and assayed repeatedly to determine Collip units/mg.

The general procedure for carrying out the assay was as follows: At least two levels of solutions of standard and unknown were prepared so that 0.5 ml. injected subcutaneously 3 times a day for 3 days produced upon autopsy, on the fourth day, ovarian weights falling within the previously determined response range of the rats. The average ovarian weight of one level of unknown must fall within the response range registered by the standard for that assay. The unknown's percentage of the standard was calculated. This percentage was multiplied by the Collip unit level at which the standard was run to get the Collip units/mg. of the unknown.

For the bioassay of the lactogenic hormone activity, pigeons 2½ months old from start of incubation were used. The international unit represents 0.1 mg. of the international standard preparation. The procedure is briefly: Solutions of two levels of standard and two levels of unknown were prepared. Deep intramuscular injections of 0.5 ml. were given daily on alternate sides of the breast bone for 4 days. The pigeons were sacrificed the following day. The crop glands were removed and weighed. The unknown's percentage of the standard was calculated. From this percentage, the potency of the unknown was calculated.

During the first part of the work on extraction of whole sheep pituitary glands for hormones, it was impossible to get a sufficient quantity of pituitary glands to carry out an adequate number of extraction experiments on the same material. From time to time over a period of several years, whole sheep pituitary glands were collected on the killing floor, and immediately placed on solid  $CO_2$ . These frozen glands were ground in a large hammer mill to a powder resembling coarse corn meal in consistency. The pulp was stored in the frozen state until it could be used.

Originally 250-g. portions of this frozen pulp were extracted in 40% alcoholic acetate buffers at pH 3.5, 4.0, 4.5, 5.5, and 6.0, and at ionic strength ( $I/2$ ) of 0.05, 0.15, 0.50, and 1.0 for each pH with the exception of 3.5 in which case ionic strength of 0.05 and 0.15 only were used. It was assumed that the frozen tissue pulp contained 75% water and that the ionic strength of the aqueous phase in the tissue was 0.15. The 250 g. of tissue pulp were extracted with 500 ml. solvent, such that the final mixture was acetate buffered at the pH values and ionic strengths indicated above, and contained 40% ethanol. After extracting for 24 hr. at  $-5^\circ C$ . with mechanical stirring, the extracts were centrifuged. The supernatants were filtered through coarse filter



paper and saved. The residue was extracted the second time with 500 ml. of 40% alcoholic acetate buffer of proper pH and ionic strength for 24 hr. at  $-5^{\circ}\text{C}$ . The extract was centrifuged, the supernatant combined with the first supernatant, and the tissue residue discarded. Originally the whole combined extract was dialyzed against distilled water until salt- and alcohol-free. The dialyzed suspension was clarified by centrifugation and filtered. The filtrate was lyophilized. The dry powder was submitted for bioassay. It was later found that the entire gonadotropic potency was precipitated by rendering the combined 40% alcohol extracts 80% with respect to ethanol. The potent precipitates were then suspended in a minimum of distilled water and dialyzed against distilled water in cellulose casings. The dialyzed suspensions were centrifuged. The clear, potent supernatants were lyophilized, and the inactive residues were lyophilized or acetone-dried. Both dry powders were submitted for bioassay.

Later, when more glands became available, a large quantity of carefully prepared acetone sheep pituitary powder was obtained. In view of the fact that all early work had been done with tissue of varying quality, an entire extraction series was carried out using 35 g. of the above acetone powder at each pH and ionic strength studied. As before, 40% alcoholic acetate buffer was used. The powder was extracted the first time with 300 ml. of the buffer and the second time with 200 ml. of the buffer. The gonadotropic potency was precipitated in each case by rendering the combined supernatants 80% with respect to ethanol. The precipitate in each case was suspended in a minimum of distilled water and dialyzed in cellulose casings against distilled water. The dialyzed suspension was centrifuged. The clear supernatant, potent in gonadotropin, was lyophilized. The inactive residue was either lyophilized or acetone-dried. The powders were then submitted for bioassay.

Due to the buffering action of pituitary tissue it was always necessary to adjust the pH during the first extraction by adding glacial acetic acid slowly.

## RESULTS

The results of the extraction series using fresh tissue indicated that the optimum conditions for extracting gonadotropin were pH 4.5,  $\Gamma/2$  0.50. Figure 1 shows the results of a limited extraction series using fresh frozen tissue. The total gonadotropic activity obtained per gram of frozen tissue is plotted against pH values. Each graph represents an ionic strength, *i.e.*, 0.05, 0.15, 0.50, and 1.00. On the basis of this finding, several large batches of sheep glands were processed by extracting with 40% alcoholic acetate buffer, pH 4.5,  $\Gamma/2$  0.50, and precipitating the gonadotropin by rendering the extracts 80% with respect to ethanol. The precipitate after centrifugation was suspended in distilled water, dialyzed against distilled water, and the soluble portion lyophilized. Exceptionally high potency material was obtained in the first several runs using acetone powder in some cases and

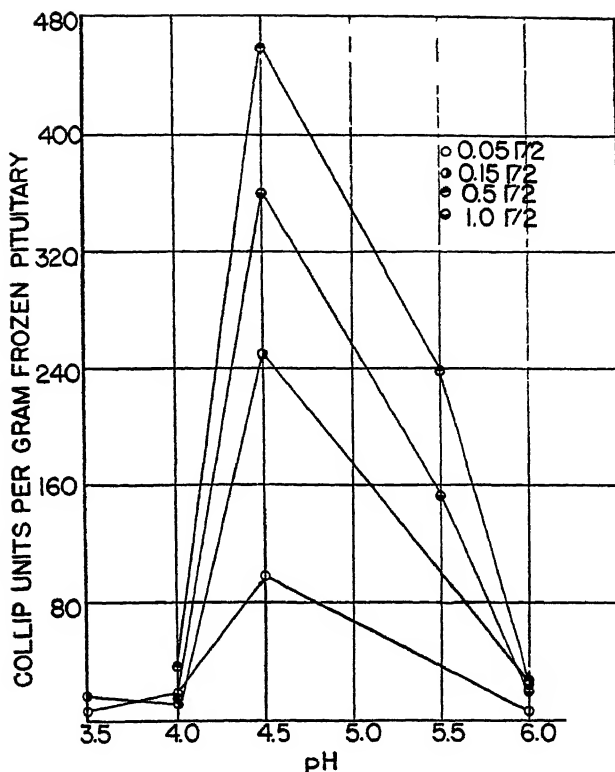


FIG. 1. Extraction series with frozen pituitary tissue.

lyophilized glands in others. Results of the first four runs are given in Table I.

The results of the more recent and more carefully executed extraction series using the same acetone powder for the whole series are given in Table II. The results indicate that pH 5.0,  $I/2$  0.5, and pH 4.5,  $I/2$  0.15 are the optimum conditions for extracting the gonadotropin from sheep acetone pituitary powder. The extraction at pH 4.5 and pH 5.0 were made in duplicate and indicate reasonable confirmation. Figure 2 gives the broken-line graphs for total gonadotropic activity obtained per gram of acetone powder plotted against pH at ionic strengths 0.15, 0.50, and 1.00.

It was found upon rendering some of the 80% alcoholic superna-

tants from the gonadotropin precipitate 30 to 35% with respect to acetone, a precipitate formed and settled out. This precipitate could be centrifuged very easily and when it was suspended in distilled water and dialyzed against distilled water separated into a water-soluble and water-insoluble fraction. Upon drying, the water-insoluble fraction contained high lactogenic potency.

A 250-g. portion of sheep anterior pituitary acetone powder was extracted with 2 l. of 40% alcoholic acetate buffer at pH 4.5,  $\Gamma/2$  0.5 at  $-5^{\circ}\text{C}$ . for 24 hr. The extract was centrifuged off and filtered. The residue was extracted with 1500 ml. of the same buffer for 24 hr. at  $-5^{\circ}\text{C}$ . The extract was centrifuged off, filtered, and combined with the first extract. The tissue residue was discarded. The combined extracts consisting of 2865 ml. were rendered 80% with respect to alcohol by

TABLE I  
*Results with Extraction of Large Quantities*

Starting material	Product	Potency	Yield in terms of starting material
	<i>g</i>	<i>C U./mg.</i>	<i>C U./g.</i>
2.23 kg. acetone powder	11.2	274	1375
2.69 kg. lyophilized glands	20.6	161	1230
6.0 kg. acetone powder	41.9	150	1047
6.12 kg. lyophilized glands	58	185	1753

adding 8000 ml. 95% ethyl alcohol through a capillary with constant stirring at  $-5^{\circ}\text{C}$ . The precipitate was allowed to settle 2 days. This precipitate after centrifugation was suspended in distilled water and dialyzed against distilled water. The soluble portion after dialysis was lyophilized and yielded powder weighing 1.8 g. This material assayed only 12.5 Collip units/mg. The insoluble residue was inactive.

The 80% alcoholic supernatant from the gonadotropin precipitate consisting of 10.82 l. was rendered 30% with respect to acetone by adding 4.5 l. pure acetone at  $-5^{\circ}\text{C}$ . The precipitate which formed was centrifuged off, suspended in distilled water, and dialyzed against distilled water. During dialysis, the precipitate separated into water-soluble and water-insoluble fractions. The soluble portion, after clarification by centrifugation, was lyophilized. The insoluble residue was dried with acetone. When assayed for lactogenic hormone, the soluble

fraction weighing 1.596 g. was inactive (2.5 units mg.). The insoluble fraction weighing 5.033 g. assayed approximately 46 units mg. Subsequent assays have shown it to be somewhat less potent, but still very high.

TABLE II  
*Extraction Series on Sheep Pituitary Acetone Powder*

pH	r/2	Product	Potency	Total yield	Yield in terms of Acetone powder
		mg.	C.U.	C.U.	C.U./g
3.5	0.15	182	27.4	4,986	143.0
3.5	0.50	194	12.5	2,425	69.4
3.5	1.00	158	9.2	1,459	41.7
4.0	0.15	98	138.0	13,524	386.0
4.0	0.50	242	47.2	11,422	326.0
4.0	1.00	274	14.4	3,945	113.0
4.5	0.15	252	218.0	54,936	1,569.6
		172	224.0	38,528	1,101.0
4.5	0.50	290	64.5	18,705	534.0
		247	73.6	18,179	520.0
4.5	1.00	960	15.5	14,880	425.0
		462	16.5	7,603	217.0
5.0	0.15	114	63.8	7,273	208.0
		140	57.5	8,050	230.0
5.0	0.50	217	266.0	57,722	1,650.0
		208	218.0	45,344	1,295.0
5.0	1.00	176	162.0	28,512	814.0
		198	124.0	24,552	700.0
5.5	0.15	105	122.0	12,810	366.0
5.5	0.50	346	38.0	13,148	376.0
5.5	1.00	250	64.0	16,000	457.0
6.0	0.15	95	41.5	3,942	113.0
6.0	0.50	597	15.5	9,253	264.0
6.0	1.00	527	23.5	12,384	354.0

Five gallons of the 80% alcoholic centrifugate and 5 gal. of the 80% alcoholic suspension from a large gonadotropin run, using approximately 11 kg. whole sheep pituitary powder, were examined for lactogenic activity. The centrifugate and the suspension were centrifuged, and rendered 35% with respect to acetone as in the previous experiment. The precipitates in both cases were centrifuged off, suspended in distilled water, and dialyzed against distilled water. The

soluble fractions after being lyophilized were found to be inactive. The insoluble fractions were lyophilized and found to be highly active. The product from the 80% alcohol centrifugate weighing 2.366 g. assayed  $40.0 \pm 27.1$  I.U./mg. The product from the 80% alcoholic centrifugate weighing 2.315 g. assayed  $19 \pm 4.5$  I.U./mg. In view of the inaccuracy

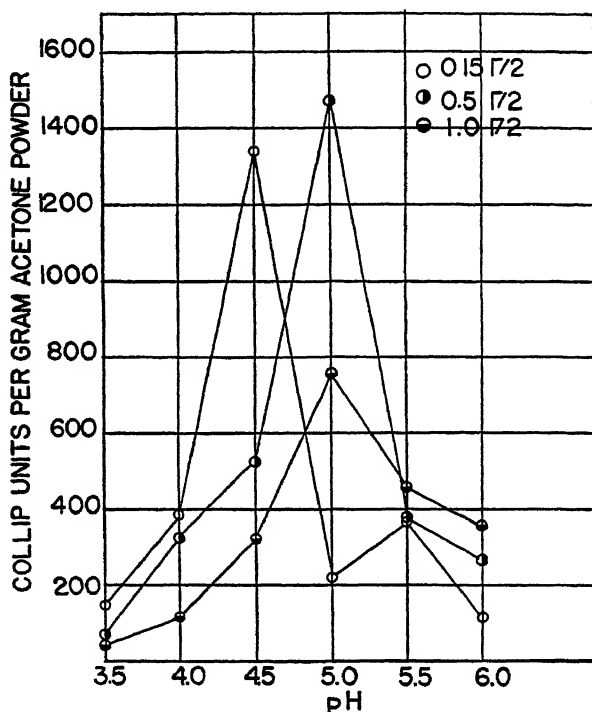


FIG. 2. Extraction series with pituitary acetone powder.

of the assay method, both products can be considered to have essentially the same potency, representing a highly purified lactogenic hormone. The fraction containing the gonadotropin assayed 64 Collip units/mg.

#### DISCUSSION

In spite of the large amount of data that were collected on extracting frozen pituitary tissue under varying conditions of pH and ionic strength with 40% alcoholic acetate buffer, considerable variation was

experienced. The starting material was not the same in all these experiments since the pituitaries were collected at different seasons of the year. At certain seasons, lamb pituitaries predominated over the pituitaries of older sheep. It was found that the gonadotropin content was highest in the lamb pituitaries. In the large runs, considerable variation was experienced in the amount of gonadotropin obtained both in potency of the product and in quantity of the product. In most instances, this could be traced to considerable variation in the starting material, whether in the form of acetone powder or lyophilized glands.

Because of this variation, the extraction series using the same acetone powder for all experiments was undertaken. It is felt that this series presents a consistent picture in view of the fact that the conditions showing maximum potency could be repeated. Extraction of the tissue at optimum conditions was considered complete since no significant amount of gonadotropin could be detected in the tissue residues. It has been found that the tissue residues after gonadotropic extraction by the above procedures contain appreciable quantities of adrenocorticotropin (ACTH).

On casual inspection of Figs. 1 and 2, one might find difficulty in reconciling the data which indicate that in the fresh tissue the optimum pH is independent of ionic strength, whereas, in acetone powder the optimum pH differs for each ionic strength. This apparent discrepancy is reconciled if one realizes that acetone powder has very different solubility behavior from either fresh tissue or lyophilized tissue, due in part to difference in lipide content. The lipides and lipoproteins affect materially the solubility behavior of proteins.

Although the most potent preparations of gonadotropin obtained by the methods outlined above contained no significant contamination with adrenocorticotropin or thyrotropin, the preparations were not found to be homogeneous electrophoretically or ultracentrifugally. It was assumed, therefore, that these potent preparations were mixtures of the so-called FSH and LH fractions, and possibly some inactive impurities.

Additional work has been and is being carried out on the refractionation of these potent powders, and careful assay work is being carried out also to differentiate if possible between LH and FSH activity.

Preliminary experiments with hypophysectomized rats indicated that they offered no advantage over immature intact animals for the bioassay of gonadotropin preparations under various conditions of

extraction. It is realized, however, that as greater purification of FSH and LH is attained more careful assay work with hypophysectomized animals will be necessary. Only immature rats were used for the data presented in this paper. The Collip assay is primarily an assay for FSH activity. If, however, LH is present, the assay is greatly influenced because of the production of corpora lutea. The procedure then becomes an assay for a mixture of FSH and LH. To assay for pure FSH and pure LH necessitates careful fractionation and assay of all fractions by several different assay methods in order to rule out augmentation effects of one factor on another. In the above extraction studies, interest was centered in spotting over-all gonadotropic activity with the idea of submitting these active fractions to more careful refractionation and assay examination later.

From a chemical standpoint, the entire process is unique in that extraction is carried out at the pH of minimum solubility of FSH and LH at elevated ionic strengths. Apparently the "three gonadotropins," FSH, LH, and prolactin only are extracted in the process with the exclusion of other anterior pituitary hormones, leaving recoverable ACTH in the tissue residue. The prolactin can be quantitatively separated from the FSH-LH mixture.

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#### SUMMARY AND CONCLUSIONS

A method for the extraction of the "three gonadotropins," follicle stimulating hormone (FSH), luteinizing hormone (LH), and prolactin, in high yield from sheep pituitaries using alcoholic acetate buffers has been described.

The lactogenic hormone has been separated in a highly purified state.

The process lends itself well to large-scale production of the gonadotropic and lactogenic hormones.

The gonadotropin, although free from measurable amounts of other pituitary hormones is not homogeneous with regard to physicochemical criteria.

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# The Metabolism of Stem Tissue During Growth and Its Inhibition. I. Carbohydrates<sup>1</sup>

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## INTRODUCTION

It has been well established in recent years, mainly through studies of the *Avena* coleoptile, that plant growth by cell enlargement, and the action of auxin in promoting such growth, is intimately related to certain aspects of cellular metabolism (14). It has become increasingly clear, therefore, that further understanding of the relation between growth and metabolism is dependent on an investigation of the fate of the major metabolites during growth and the inhibition of growth. The experiments to be described in this series of papers constitute such an investigation.

Although most of the previous work on this subject has been done using the *Avena* coleoptile, it is in some ways not the best material for metabolite studies. In order for auxin to cause appreciable increase of growth an external source of carbohydrate or organic acid must be supplied (5, 12, 17), a complication which makes it difficult to interpret any subsequent analysis for these metabolites in the tissue. Isolated pea stem sections, on the other hand, do not require carbohydrates to be added in order to give good growth. They have been well studied and widely used in growth tests (6, 17, 21) and their growth behavior closely resembles that of *Avena* coleoptile sections (15, 18). The significant differences, mainly that added metabolites do not greatly enhance growth and that their sensitivity to auxin and inhibitors is in general less than that of coleoptiles, can be explained by assuming that the pea stem sections contain an integral supply of all the metabolites necessary for the somewhat limited growth of which they are capable. As far as sugars are concerned this is borne out by the high figures for reducing sugar in the analyses given below; in the case of organic acids it has already been shown (15) that the pea stem contains more than the *Avena* coleoptile.

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In these experiments, therefore, isolated stem sections of etiolated *Pisum* seedlings were used to study the effects of auxin on growth, respiration, and the formation and disappearance of various metabolites. The effects of three enzyme poisons which have previously been found to inhibit growth were also studied; these were iodoacetate (5, 17), fluoride (1, 2, 19) and arsenite (18). Part of these results have been published in preliminary form elsewhere (3).

### GENERAL METHODS AND MATERIALS

Seeds of *Pisum sativum* (Var. Alaska) were soaked in tap water for 6-8 hr., then germinated and allowed to grow on specially designed plastic holders over dishes of tap water. The germination and growth of plants as well as all subsequent treatments were done in a darkroom maintained at 25°C. and 87% relative humidity. Growth was in total darkness and all operations were carried out with a minimum of red light (Corning Signal red filter) in order to avoid the possibility of photosynthesis or any effects of light on growth.

At the end of 7 days, 20.0-mm. sections were cut from the most apical part of the third internode, the most rapidly growing part of the plant at this time. Plants which had started the fourth internode (beyond a length of about 3 mm.) were rejected. After washing, the sections were divided into random samples of 15 sections each, blotted dry, and weighed. The sections were then allowed to grow for 24 hr. by floating on the surface of the various test solutions, so that they were well aerated.

Pure 3-indoleacetic acid was used as the auxin source throughout. All solutions were brought to pH 6.5 with KOH or H<sub>2</sub>SO<sub>4</sub>. Solutions were kept at 5°C. except while actually in use and were prepared fresh every few weeks. The auxin controls grew in a solution of 1 mg./l. 3-indoleacetic acid in water; in all experiments the inhibitors were used in the presence of this same concentration of indoleacetic acid as auxin.

At the end of the 24-hr. growth period the samples were blotted and weighed again to the nearest milligram and the individual sections were measured to the nearest millimeter. The samples were then immediately subjected to the various metabolite analyses.

### EXPERIMENTAL

#### *Growth*

Growth was measured both as increase in length and as increase in fresh weight. In water the 20-mm. sections increased in length by 4.0 mm. or 20% and in fresh weight by 22.2%. In auxin (1 mg. l. 3-indoleacetic acid) the sections elongated 10.2 mm. or 51% and increased in fresh weight by 58.8% (average of 17 experiments). The elongation in individual experiments in auxin varied between 47% and 55%. Thus the extent of growth is not large but it is very reproducible.

The final lengths of the sections after growth in auxin and serial dilutions of the three inhibitors mentioned above are shown in Fig. 1 for iodoacetate, in Fig. 2 (left) for arsenite, and in Fig. 2 (right) for fluoride. It will be seen that the concentrations necessary to inhibit growth are very different, arsenite being the most effective and fluoride the least. However, the absolute concentrations are of rela-

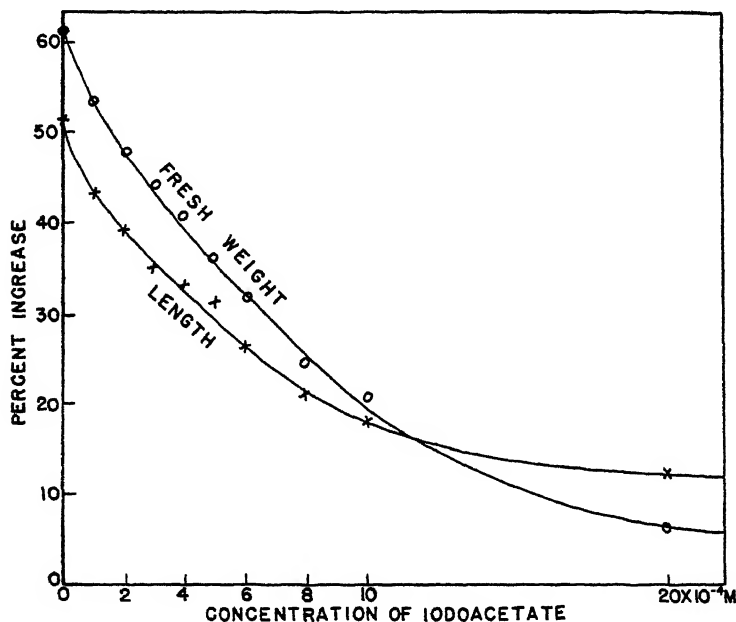


FIG. 1. The effect of iodoacetate on the growth of pea-stem sections, expressed as increase in length and in fresh weight. Average initial fresh weight of the 15 section samples was 678.8 mg., length 20.0 mm. All points represent the average of four or more samples of 15 sections each. All concentrations of iodoacetate were used in the presence of 1 mg./l. indoleacetic acid.

tively little importance, since what was desired was to study the effect on metabolism which accompanies a given degree of inhibition of growth. For this purpose the concentration necessary to produce 50% growth inhibition was obtained from Figs. 1 and 2, and many of the subsequent determinations were made only at these concentrations:  $6 \times 10^{-4}$  for iodoacetate,  $10^{-4}$  for arsenite and  $5 \times 10^{-3}$  for fluoride.

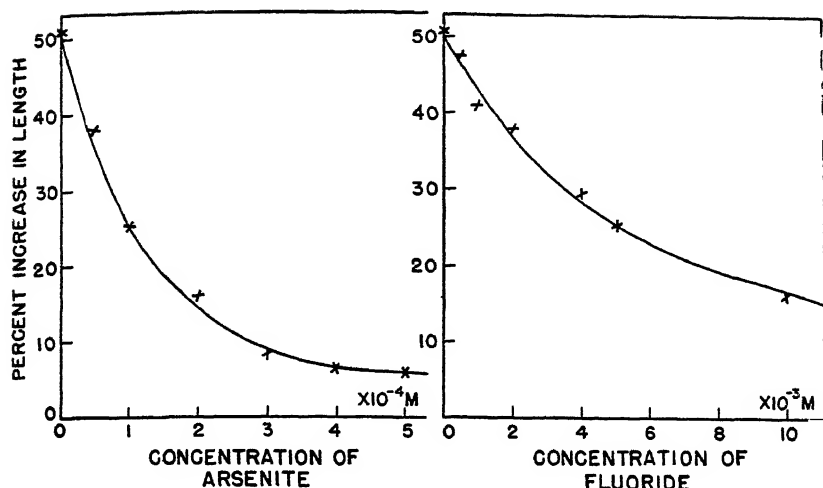


FIG. 2. The effect of arsenite (left) and fluoride (right) on the elongation of pea-stem sections in the presence of 1 mg./l. indoleacetic acid. Material and treatments as in Fig. 1.

In Fig. 1 the increase in fresh weight is compared with the increase in length in sections inhibited with iodoacetate. It is seen that these two curves are quite closely parallel up to about  $10^{-3} M$ . Thus, over the concentration range at which this inhibitor is normally used, fresh weight and length are completely comparable as indexes of growth.

### *Growth Rate*

The rates of growth of the sections in water, auxin, and auxin plus the 50% inhibiting concentration of iodoacetate were determined in special experiments by length measurements at 2-hr. intervals over the active growth period. Shadowgraphs were taken of the samples on plates sensitive to the red light of the darkroom. The section lengths were then determined by projecting the shadowgraphs in a slide lantern and measuring the images with a meter rule.

The growth rate curves obtained in this way are shown in Fig. 3. The curve in auxin agrees roughly with that given by Galston and Hand (6). It will be seen that growth is very rapid and increasing in rate for the first 4 hr. The rate then falls off rapidly and growth has ceased entirely at the end of 12 hr. Analyses and all growth measure-

ments (except those of Fig. 3) were therefore made after 24 hr., when all growth was over. Thus the sections grew in darkness throughout.

### *Effect of Sugars on Growth*

In view of the well known and very striking effect of sucrose on the growth of the *Avena* coleoptile (12), it was first considered important to investigate the effect of sugars on pea-stem sections. Length, fresh-weight changes, and dry-weight changes were measured on sections after growth in solutions to which varying amounts of sucrose and glucose were added in addition to the other constituents being studied. Table I shows the results of these determinations.

As was expected, no growth stimulation by these sugars was found. On the other hand, there are quite notable effects on fresh weight and dry weight. Sections growing in solutions containing auxin and sugar take up a greater amount of water as compared with similar treatment

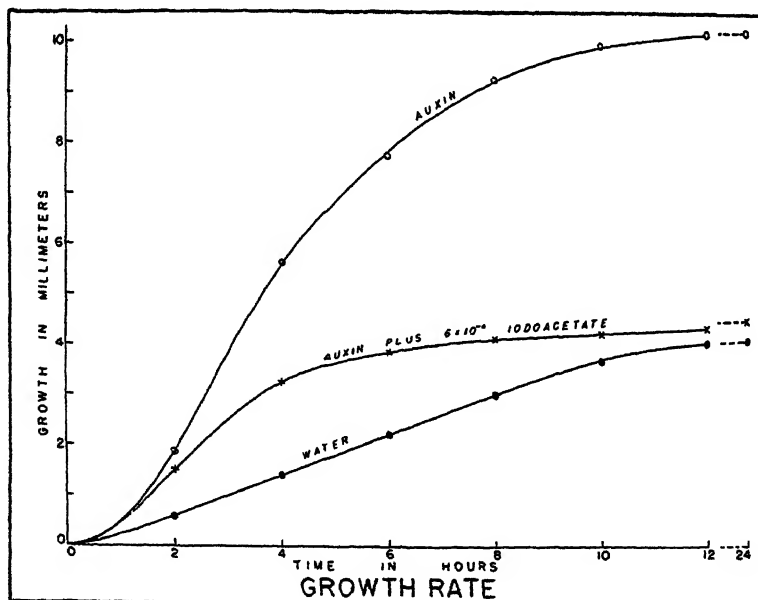


FIG. 3. Time course of growth of pea-stem sections. Each set of points represents the average length of duplicate samples of 15 sections each. Initial length of sections was 20.0 mm.

TABLE I  
Effect of Sugars on Growth of Pea-Stem Sections<sup>a</sup>

Growth solution	Increase in		Dry-weight <sup>d</sup> change
	Length <sup>b</sup>	Fresh weight <sup>c</sup>	
Water	20	22	-11.0
Auxin 1 mg. l.	51	59	-11.0
1% Sucrose = 0.029 M	19	18	- 1.7
1% Glucose = 0.056 M	17	16	- 0.8
Auxin plus 1% sucrose	52	68	+13.5
Auxin plus 6 × 10 <sup>-4</sup> M iodoacetate	26	23	-17.6
Auxin plus 6 × 10 <sup>-4</sup> M iodoacetate plus:			
3% Sucrose	21	23	+ 0.9
1% Sucrose	23	28	- 7.0
0.33% Sucrose	25	28	-11.7
3% Glucose	19	23	+ 1.2
1% Glucose	22	25	- 7.2
0.33% Glucose	25	26	-12.5

<sup>a</sup> All data are the average for two samples of 15 sections each.

<sup>b</sup> Initial length was 20.0 mm.

<sup>c</sup> The average initial fresh weight of the 15 section samples was 678.8 mg.

<sup>d</sup> The initial dry weight was calculated from the initial fresh weight by use of the factor 6.70%.

in the absence of sugar. The effect of sugar on dry-weight change—an actual increase rather than a decrease (see below)—is similar to that observed with the *Avena* coleoptile growing in auxin-sucrose solutions (Thimann and W. Bonner, unpublished) and indicates that some sugar does enter the sections.

The negligible effect of sugar on elongation contrasts strongly with the results of Galston and Hand (6) who record a 59% ± 3.5% increase in growth of 5-mm. pea-stem sections when 2% sucrose was added. Extensive experience in this laboratory, of which Table I is only an example, had shown, however, that sucrose exerts little or no effect on the growth of pea sections; indeed it was for this reason that pea stems were selected for the present experiments.

All of the experiments of Galston and Hand were done in the presence of a phosphate buffer (concentration unspecified). Now Spear and Thimann (13) have shown that the combination of sucrose and potassium phosphate stimulates growth as measured by pea curvatures. In view of these observations, the relation between added

sugar and growth was reinvestigated. Various combinations of sucrose, potassium phosphate buffer, and potassium chloride were added to the growth solutions. The results of these experiments are shown in Table II. It will be seen that there is no growth promotion by sucrose under our conditions. There is, however, a very small effect of potassium phosphate which can apparently be duplicated by potassium chloride. This effect of potassium agrees with other data from this laboratory (20).

One outstanding point in which the methods of Galston and Hand differ from ours is that they cut their sections 5 mm. long, while ours are 20 mm. long. Since the difference in results might be correlated with this different initial length, the growth

TABLE II  
*Effects of Sucrose and Potassium Salts on Elongation*

Treatment	Growth as per cent increase in length <sup>a</sup>			
	Sucrose concentration, %			
	0	0.3	1.0	3.0
Water	13.0	14.5	15.0	—
KH <sub>2</sub> PO <sub>4</sub> (adjusted to pH 6.0 with KOH)				
$4 \times 10^{-3} M$	17.0	16.5	17.0	—
$10^{-2} M$	16.0	18.0	16.5	—
KCl $4 \times 10^{-3} M$	16.0		19.0	—
Auxin	48.5	47.0	43.0	—
Auxin plus KH <sub>2</sub> PO <sub>4</sub>				
$2 \times 10^{-3} M$		45.5	49.5	42.5
$4 \times 10^{-3} M$	51.5	50.0	48.5	40.0
$10^{-2} M$	52.0	51.5	44.5	36.5
Auxin plus KCl				
$4 \times 10^{-3} M$	50.5		51.5	

<sup>a</sup> Data are the average for single samples of 15 sections each. Initial length 20.0 mm.

of 5-mm. sections was studied. In experiments based on 4 samples of 20 sections each, the sections in auxin, 1 mg./l., grew 51.9%, which agrees perfectly with the data for 20-mm. sections in Tables I and II. With 1% sucrose growth was 56.8%; in auxin plus potassium phosphate buffer, 0.004 M, growth was 55.5% and in auxin plus KCl, 0.004 M, growth was 55.7%. Sucrose, 1%, raised these last two figures to 63.6 and 63.7%, respectively. The increase due to 1% sucrose amounts to 5% in the absence of potassium and 7-8% in its presence. In all cases the stimulation due to 2% sucrose was much less still. Thus there is a slight effect of sucrose on 5-mm. sections, and the effect is increased by the presence of potassium; even so, however, it is smaller than that reported by Galston and Hand.

TABLE III  
*Changes in Dry Weight During Growth*

Treatment	Dry-weight increase, %
Water control	11.0 <sup>a</sup>
Auxin 1 mg./l.	11.0 <sup>a</sup>
Auxin plus:	
$10^{-4}$ M arsenite	11.6 <sup>b</sup>
$2 \times 10^{-4}$ M arsenite	11.9
$4 \times 10^{-4}$ M arsenite	11.9
$5 \times 10^{-4}$ M arsenite	11.5
$5 \times 10^{-4}$ M fluoride	13.5
$10^{-3}$ M fluoride	13.7
$2 \times 10^{-3}$ M fluoride	13.5
$4 \times 10^{-3}$ M fluoride	13.7
$5 \times 10^{-3}$ M fluoride	12.2 <sup>b</sup>
$6 \times 10^{-4}$ M iodoacetate (cf. Fig. 4)	17.6 <sup>b</sup>

<sup>a</sup> Average of 13 samples of 15 sections each.

<sup>b</sup> Average of 8 samples. All other data are the average of 2 or more samples.

It is to be noted that the amount of growth recorded by Galston and Hand in the presence of 10 mg./l. of auxin and 2% sucrose is 59% in their Table 2, and 45% in their Fig. 9.<sup>2</sup> The average of these, 52%, agrees well with our results obtained in the absence of sugar. At an auxin concentration of 1 mg./l., which is that used by us, their data (Figs. 4, 6, and 7) show a percentage growth of 64, 75, and 45, respectively; presumably these experiments were all carried out in the absence of sugar, and the largest figure, 75%, is specifically stated to be for auxin "in glass-distilled water." The only other datum which appears to refer to the presence of sugar (at this auxin concentration) is that of their Fig. 9 which gives only 51% growth (cf. values above). It is clear that the spread of results between the individual experiments reported is too great to allow the conclusion that added sucrose has any large effect on growth.

### *Dry-Weight Change*

The relationship between initial dry weight and initial fresh weight was established by weighing samples of sections from each experiment immediately after cutting, then drying to constant weight at 105°C. (about 48 hr.). The ratio between the initial dry and fresh weights, averaged from 17 experiments using a total of 500 sections, was 6.70%, with individual determinations ranging from 6.4% to 7.1%. This ratio was used to calculate the initial dry weight in the growth experiments,

<sup>2</sup> The percentage values are calculated from their data in millimeters, initial length being given as 5.34 mm.



and all metabolite determinations were referred to this derived value of the initial dry weight.

Samples of sections grown for the 24-hr. period in the various growth solutions were similarly dried to constant weight and thus the changes in dry weight during growth were determined. It was found in this way that sections grown in water lose dry weight to exactly the same extent as those grown in auxin. This loss amounts to 11.0% of the initial dry weight (average of 13 experiments). The upper curve of Fig. 4 shows the effect of increasing concentration of iodoacetate on the dry weight loss, while Table III shows the effects of arsenite and fluoride. It is seen that all of the inhibitors cause a greater loss in dry weight than takes place in auxin alone, although this difference is hardly significant in the case of arsenite.

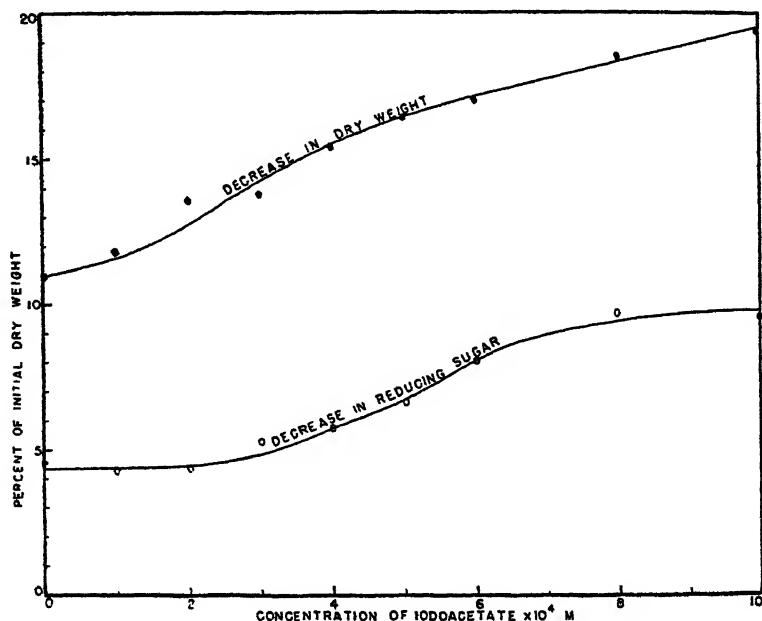


FIG. 4. The effect of iodoacetate on the dry weight (upper curve) and reducing sugar content (lower curve) of pea-stem sections. The average initial dry weight of the 15 section samples was 45.5 mg.; the initial content of reducing sugar was 17.5% of the initial dry weight. All concentrations of iodoacetate were used in the presence of 1 mg./l. indoleacetic acid.

*Reducing Sugar*

Reducing sugars were determined on samples of sections before growth and after 24 hr. of growth in the various solutions. The crushed sections were extracted with boiling 80% aqueous ethanol, the solution cleared with neutral lead acetate, delead with sodium oxalate, and the reducing sugars finally titrated by the Shaffer-Hartman-Somogyi procedure as described by Harrow (7). The method was standardized against c.p. glucose and proved entirely reproducible.

TABLE IV  
*Reducing Sugars*

Treatment	Reducing Sugar as per cent of Initial Dry Weight Content	Change
Initial	17.5 <sup>a</sup>	—
Water control	12.8 <sup>a</sup>	— 4.7
Auxin 1 mg./l.	12.9 <sup>a</sup>	— 4.6
Auxin plus:		
10 <sup>-6</sup> M arsenite	13.6	— 3.9
10 <sup>-4</sup> M arsenite	10.8 <sup>a</sup>	— 6.7
10 <sup>-3</sup> M arsenite	7.8	— 9.7
10 <sup>-4</sup> M fluoride	15.0	— 2.5
5 × 10 <sup>-4</sup> M fluoride	14.9	— 2.6
10 <sup>-3</sup> M fluoride	11.9	— 5.6
5 × 10 <sup>-3</sup> M fluoride	7.2 <sup>a</sup>	— 10.3
10 <sup>-2</sup> M fluoride	6.1	— 11.5
6 × 10 <sup>-4</sup> M iodoacetate (cf. Fig. 4)	9.5 <sup>a</sup>	— 8.0

<sup>a</sup> Average of duplicate determinations on 8 or more samples of 15 sections. All other data are the average of duplicate determinations of 2 or more samples.

The results of these determinations are shown in Table IV, and for iodoacetate in the lower curve of Fig. 4. It is seen that, although there is a consumption of reducing sugar during growth stimulated by auxin, there is a similar utilization in the water controls which grow only one-third as much. Thus the consumption of total dry weight and of reducing sugar are essentially the same with and without auxin. In each case the consumption of reducing sugar accounts for about one-half the loss in dry weight.

In the presence of the inhibitors the loss of reducing sugar is even greater, increasing with increasing inhibition of growth. Thus it is apparent that the amount of growth accomplished does not depend on the amount of reducing sugar consumed. That the growth inhibition is not

itself *due* to disappearance of reducing sugar is shown by the experiments described in Tables I and II in which addition of sugar gave no increase in growth.

### *Sucrose*

Attempts were made to determine sucrose by the invertase method but the results were erratic and not reproducible, probably due to a combination of (a) a small amount of sucrose in comparison to reducing sugar, and (b) a high incidence of microbial contamination. It was found, however, that a modification of the Seliwanoff resorcinol color test for fructose (11) was applicable to the quantitative estimation of sucrose. Meeuse (private communication) has shown that this color

TABLE V  
*Changes in Sucrose during Growth*

Treatment	Growth <sup>a</sup>	Sucrose as per cent initial dry weight <sup>b</sup>	
		Content	Change
Initial	—	5.59	—
Water control	20.0	2.41	-3.18
Auxin 1 mg. l.	51.0	1.61	-3.98
Auxin plus:			
$6 \times 10^{-4}$ M iodoacetate	25.5	2.35	-3.24
$10^{-4}$ M arsenite	26.5	1.46	-4.13
$5 \times 10^{-3}$ M fluoride	25.5	1.44	-4.15

<sup>a</sup> Derived from Figs. 1 and 2.

<sup>b</sup> Average of duplicate determinations on 2 samples of 30 sections each.

reaction determines both free and combined fructose. This fact, coupled with the observation that intact 7-day-old pea seedlings contain no appreciable free fructose, allowed Meeuse to use this method as an analysis for sucrose. The values obtained by him using this method were in close agreement with those obtained by very carefully controlled sterile invertase determinations.

The method was first calibrated against C.P. sucrose; the relationship between the amount of sucrose in the sample and the colorimeter reading in the Klett, using filter K56, was linear from 0.25 at least up to 1.5 mg. Thus, the method gives reliable results over the concentration range used.

The results of these sucrose determinations are shown in Table V. The sucrose content is evidently considerably less than the reducing sugar content of the sections, but the changes in the two sugars during growth and inhibition show some similarities. However, sucrose disappears to a greater extent in auxin than in water. When growth is inhibited by arsenite or fluoride there is (as with reducing sugars) an increased utilization of sucrose. Iodoacetate appears to reverse the increased utilization caused by auxin. However, evidence to be presented in a later paper indicates that some free fructose may be present in the iodoacetate-inhibited sections, thus throwing some doubt on this high value for the sucrose content. This consideration does not hold for the other treatments, since the initial samples contain no fructose, and both the sucrose and the reducing sugar values are decreased below that of the initial sample.

Consideration of the changes in sucrose in the water and auxin controls only would suggest that the utilization of this sugar, in contrast to that of reducing sugar, is characteristic of growth. However, the effects of the inhibitors, which increase this utilization, indicate a more complex relationship.

### *Phosphate Esters, Starch, and Dextrins*

Analyses for inorganic and total phosphate showed conclusively that the sugar which disappears during growth does not accumulate as phosphate ester intermediates. These analyses were made by the method of Pregl (9) for total phosphorus using incinerated intact sections. The inorganic phosphorus was determined on fresh sections, ground with sand to a fine pulp at room temperature, by Umbreit's adaptation of the Fiske-SubbaRow method (22). The results of these determinations (Table VI) show clearly that there is no appreciable

TABLE VI  
*Inorganic and Total Phosphate*

Treatment	Phosphate, per cent of initial dry weight <sup>a</sup>	
	Inorganic	Total
Initial	0.25	0.27
Water control	0.28	0.28
Auxin 1 mg./l.	0.29	0.29
Auxin plus: 6 × 10 <sup>-4</sup> M iodoacetate	0.23	0.24

<sup>a</sup> Average of 4 samples of 15 sections each.

amount of organically combined phosphorus and hence that no sugar disappears by accumulating as phosphate esters. This conclusion was reported earlier (3) on the basis of acid and alkali hydrolysis experiments. The possibility that catalytic amounts of organic phosphorus compounds are active in carbohydrate conversion is of course not excluded. Even if there were some hydrolysis during the grinding of the sections, the maximum amount of sugar which could be combined with the total phosphorus is less than 0.5% of the initial dry weight, or less than one-tenth of the decrease to be accounted for.

TABLE VII

*Change in Reducing Sugar on Hydrolysis with 1% HCl for 1 Hr. at 15 Lb.*

Treatment	Per cent of initial dry weight	
	Increase in reducing sugar <sup>a</sup>	Sucrose <sup>b</sup> ÷ 2
Initial	2.24	2.79
Water control	1.18	1.20
Auxin 1 mg./l.	0.60	0.80
Auxin plus: 6 × 10 <sup>-4</sup> M iodoacetate	0.91	1.17

<sup>a</sup> Average of 2 samples of 15 sections each.

<sup>b</sup> Derived from Table V. The fructose half of the sucrose would be destroyed under the conditions of hydrolysis so that only half the weight of sucrose appears as reducing sugar on hydrolysis.

Analyses by the diastase method showed that the sections contained no detectable amount of starch and this was confirmed by the failure of the tissue to produce any color with iodine. The failure of the iodine color reaction serves also to indicate the absence of dextrans. This was further confirmed by reducing-sugar analyses of tissue extracts after hydrolysis with 1% HCl, autoclaved for 1 hr. at 15 lb. pressure. Table VII shows that the increase in reducing sugar due to the hydrolysis is entirely accounted for by the sucrose in the extract. Hence there can be no appreciable amount of dextrans in this tissue.

### *Cell Wall*

The total cell wall material and its components were determined by serial extraction of the various components and weighing the residue after each extraction. The method used was a combination of that of Wirth (23) and that of Thimann and Bonner (16).

Samples of 30 sections each were grown for the 24-hr. period, then ground to a fine pulp. This pulp was extracted successively with cold water, hot water, ether, hot 0.5% ammonium oxalate, hot 2% sulfuric acid, hot 2% potassium hydroxide, and finally ether again. The material not extracted by water was considered total cell wall material; of this total, the oxalate extracts pectic substances, sulfuric acid dissolves the cell wall protein and a part of the insoluble polysaccharides classed as hemicelluloses, potassium hydroxide extracts the remainder of the hemicellulose, and ether extracts fats and waxes of the cell wall.

TABLE VIII  
*Cell Wall Constituents*

Treatment	Per cent of initial dry weight <sup>a</sup>						
	Pectin	Hemi-cellulose <sup>b</sup>	Cellulose	Ether solubles	Protein <sup>c</sup>	Total cell wall	Cell wall deposited
Initial	5.2	10.7	7.0	0.3	3.4	26.6	—
Water control	5.1	11.8	8.0	0.3	4.4	29.6	3.0
Auxin 1 mg./l.	5.5	12.6	9.1	0.2	4.3	31.7	5.1
Auxin plus:							
6 × 10 <sup>-4</sup> M iodoacetate	5.5	11.9	8.5	0.1	3.8	29.8	3.2
10 <sup>-1</sup> M arsenite	5.2	12.2	8.1	0.2	4.2	29.9	3.3
5 × 10 <sup>-3</sup> M fluoride	6.7	11.0	8.1	0.3	4.1	30.2	3.6

<sup>a</sup> Based on duplicate samples of 30 sections each.

<sup>b</sup> Obtained by subtracting the cell wall protein (column 5) from the weight of the combined KOH and H<sub>2</sub>SO<sub>4</sub> extracts.

<sup>c</sup> Calculated from the nitrogen content of the H<sub>2</sub>SO<sub>4</sub> extract by the factor 6.2.

The cell wall protein was calculated by multiplying the nitrogen of the sulfuric acid extract (determined by the micro-Kjeldahl method) by the factor 6.2. The resulting figure was subtracted from the weight of material extracted by acid and alkali to give the hemicellulose. The residue, after all extraction, is considered as cellulose, since it is unlikely that in material with such a large percentage of elongating cells any considerable amounts of lignin would be present.

The results of these determinations are presented in Table VIII. There is, as would be expected, an increase in the percentage of total cell wall during growth under all conditions. Further, this deposition of cell wall is very nearly proportional to growth. The most significant

effects of auxin and the inhibitors on the cell wall composition are shown by the changes in the polysaccharides. These insoluble carbohydrates increase only slightly in the water control, quite markedly in the presence of auxin and to an intermediate degree in the inhibited sections. Thus one of the effects of auxin on the growth of this tissue is to stimulate the deposition of carbohydrates in the cell wall. A major effect of the inhibitors is to prevent this stimulation.

TABLE IX  
*Total Polysaccharides per Unit Length of Pea-Stem Sections*

Treatment	Length (average of 15 sections)	Total polysaccharides and polyuronides <sup>a</sup>	
		As per cent of initial dry weight	Per unit length
	<i>mm</i>		<i>μg./mm</i>
Initial	20.0	22.9	34.8
Water control	24.0	24.9	31.5
Auxin 1 mg./l.	30.2	27.2	27.4
Auxin plus:			
6 × 10 <sup>-4</sup> M iodoacetate	25.1	25.9	31.3
1 × 10 <sup>-4</sup> M arsenite	23.6	25.5	32.8
5 × 10 <sup>-3</sup> M fluoride	25.1	25.8	31.2

<sup>a</sup> Data based on sum of cellulose, hemicellulose, and pectin determinations, and on 3.03 mg. dry wt./initial 20 mm. section.

Table IX shows the results of calculation of the polysaccharides per unit length of sections. The initial value of 34.8 μg./mm. may be compared with 4.1 μg./mm. of the *Avena* coleoptile (16). Table IX shows that although polysaccharides are deposited during growth, the amount per unit length falls off—by 20% of the initial value in auxin, and by 10% both in the water controls and in auxin plus each of the three inhibitors. Thus it would appear that new cell wall of elongating cells is made up partly of new structural material but partly at least by a stretching of the existing cell wall.

Perhaps the clearest conclusion from all the above data is that the composition of the cell walls is nearly constant, and that any effects on it exerted by growth or inhibition are minor compared to the changes in simple sugar.

## DISCUSSION

The close parallel between water uptake, as measured by fresh-weight increase, and growth as measured by elongation, is worth comment. In the past these two processes have often been thought of as distinct. Thus Reinders (10), in her study of the water uptake of slices of potato and other tissues, avoids the use of the word "growth," although the process obviously is an irreversible increase in volume. The later experiments of Commoner *et al.* (4) and of Kelly (8), as compared with experiments on growth (5, 17, 18), clearly show that the effects of auxin and inhibitors are essentially the same whether water uptake or elongation is being measured. Our own data show the same thing and it is evident that the two phenomena are facets of the same process.

The S shape of the growth rate curves of Fig. 3 could be considered as indicating that some critical materials, either enzyme systems or, more probably, substrates, are being depleted and ultimately limit growth. That sugar is not limiting is shown by the results of the sugar feeding experiments (Tables I and II). However, sugar is consumed during growth and a part of this sugar is deposited as polysaccharides in the cell wall. But this deposition accounts for only one-fourth of the sugar disappearing during 24 hr. in water, and one-half of that disappearing during growth in auxin. Since the disappearance of reducing sugars is not increased by auxin it is evident that the increased cell wall is either not formed from reducing sugars, or else that the reducing sugar so consumed is continually being made up. The three inhibitors cause an increased utilization of simple sugar and at the same time decrease the deposition of cell wall carbohydrate so that the ratio of deposited to utilized carbohydrate falls to between one-fourth and one-fifth.

The simple sugar which disappears other than by deposition must either be consumed in oxidative metabolism or by conversion to other types of cell constituents. Some of it, of course, must be oxidized, but that the inhibitors could cause an increased oxidation is doubtful since all three substances are known to block carbohydrate destruction either in the oxidative or glycolytic stage. Experiments to be reported show that indeed the inhibitors do not accelerate the total rate of oxygen consumption. It must be remembered that current biochemical interpretations indicate that the reactions leading to the formation and destruc-



tion of sugars are all reversible. Hence the apparent increase in sugar consumption caused by the inhibitors could equally well be due to a decreased formation of sugars. Determinations of the changes in other metabolites, to be reported in subsequent papers, go far toward supporting the latter view.

#### ACKNOWLEDGMENT

Acknowledgment is made to Mr. Lawrence J. Kunz for carrying out some of the initial determinations of reducing sugars, particularly those with inhibitors included in Table IV. Mr. Kunz's assistance was of great value.

#### SUMMARY

Isolated sections of stems of etiolated pea seedlings have been used for a study of the relation between growth and metabolism, because their growth is not limited by added metabolites.

Growth-concentration curves have been determined for the three inhibitors, iodoacetate, arsenite, and fluoride, acting in the presence of indoleacetic acid, 1 mg./l. The 50% inhibiting concentrations of these inhibitors are, respectively,  $6 \times 10^{-4} M$ ,  $1 \times 10^{-4} M$ , and  $5 \times 10^{-3} M$ .

The growth rate at 25°C. follows a typical S-shaped curve with cessation of growth at about 12 hr. after cutting the sections. There is a very close parallel in all cases between elongation and increase of fresh weight.

Reducing sugar and sucrose are consumed during growth in water; when growth is stimulated by auxin the consumption of reducing sugar is unchanged while that of sucrose is increased by 25%. Inhibition of growth, however, caused a still greater consumption of these sugars, and this was true for all three inhibitors.

Starch, dextrans, and phosphorylated intermediates are not present in this tissue in detectable amounts.

Cell wall polysaccharides and polyuronides are deposited in parallel to the extent of growth of the sections. The proportions of the different cell-wall constituents are essentially the same after growth in water, auxin, or auxin plus inhibitors. However, the cell wall accounts for only between one-fifth and one-half of the simple sugars consumed. The increased consumption of reducing sugars caused by the inhibitors is therefore not due to polysaccharide formation.

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# The Metabolism of Stem Tissue During Growth and Its Inhibition. II. Respiration and Ether-Soluble Material<sup>1</sup>

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## INTRODUCTION

It was shown in the preceding paper (4) that during the growth of sections of etiolated pea stems in auxin solution a considerable amount of sucrose and reducing sugar is consumed. Only a small part of this is converted to polysaccharides. It was further shown that the amount of the sugars which disappears is still greater when growth is inhibited; the more complete the growth inhibition, the greater is the disappearance of sugar. Three different inhibitors, namely iodoacetate, arsenite, and fluoride, produced the same effect. It was found, as expected, that this could not be ascribed to increased deposition of cell-wall polysaccharides in the inhibited tissue. The fate of the sugar which is consumed might therefore be: (a) respiratory destruction, or (b) conversion to other, non-carbohydrate types of cell constituents. The former would require that the growth inhibitors cause an increase in respiration, while the latter would imply a more complex and far-reaching influence on metabolism.

In the present experiments these possibilities have been investigated. Measurements of respiration and determinations of the ether-soluble metabolites of the tissue during growth and inhibition are presented, and it is shown that the metabolic changes accompanying inhibition are indeed extensive.

## METHODS

The general methods for growing plants, selecting and cutting sections, and preparing solutions have been described in the preceding paper. Identical methods were used for the experiments reported here, except that the size of the tissue sample

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<sup>1</sup> This work was made possible through a grant from the Committee on Growth of the National Research Council, acting for the American Cancer Society, Inc., to whom we are much indebted.

was adjusted for convenience of analysis. For the respiration experiments, five 15-mm. sections were floated in 3.0 ml. of growth solution; for the determinations of ether-solubles, thirty 20-mm. sections were used. Indoleacetic acid, 1 mg./l., was used as auxin throughout. No sugars were added to the solutions either for growth or respiration; it was made clear in the preceding paper that added sugar has virtually no effect on the growth of pea stems. As inhibitors, iodoacetate, arsenite, and fluoride were used. Solutions were brought to pH 6.0 with  $H_2SO_4$  or KOH.

### RESPIRATION

Respiration measurements were made using standard, constant-volume Warburg respirometers [for technique see Umbreit *et al.* (13)]. The bath was maintained at 25.0°C., the temperature of the darkroom in which the growth experiments were done. Weak red lights only were used in these, as in all the other experiments. Furthermore, care was taken to shield the sections from even this weak light since, if any chlorophyll were formed, the resulting photosynthesis would mask the significance of gas exchange measurements.

Attempts were first made to determine respiration rates during the entire 24-hr. growth period, but it was found that bacterial contamination of the growth solution became so great toward the end of this period that respiration measurements were meaningless. It was decided then to carry out respiration measurements for shorter times using larger quantities of tissue. Microbial contamination was found by respiration measurements to be insignificant up to 14 hr. after the time of cutting the sections. Reference to the growth rate curves (Fig. 4 of Paper I) (4) indicated that measurements during the two following periods would be most significant: first, during the period of very rapid growth, 2-4 hr. after cutting, and second, after growth had ceased, 12-14 hr. after cutting.

### Oxygen Uptake

The tissue samples were weighed fresh immediately after cutting, and then placed in the respirometer vessels which contained 3.0 ml. of growth solution in the body of the vessel and 0.2 ml. of 20% KOH in the center well. The vessels were attached to the manometers and allowed to equilibrate in the water bath for 30-60 min. before oxygen uptake was measured. The stopcocks were closed at 2 hr. after cutting the sections and the manometers were read every 20 min. for the next 2 hr. The stopcocks were then opened and the manometer fluid returned to the reference level. After 8 hr. the stopcocks were again closed and the rate of gas exchange was followed for another 2 hr.

The results of a typical  $Q_{O_2}$  determination are shown in Fig. 1, in which are presented data on oxygen uptake in water, in auxin, and in auxin plus the 50% growth-inhibiting concentrations of iodoacetate and arsenite. It can be seen that in this experiment auxin caused a

distinct increase in the rate of oxygen uptake over that in water. As a mean of 10 similar experiments, this increase is 27% during the first period and 15% during the second.

Table I presents the results of all the  $Q_{O_2}$  determinations. The figures for water and auxin controls represent the mean of 10 experiments, those for the inhibitors each represent the mean of 2 to 6 experiments. All the experiments with inhibitors were carried out in the presence of

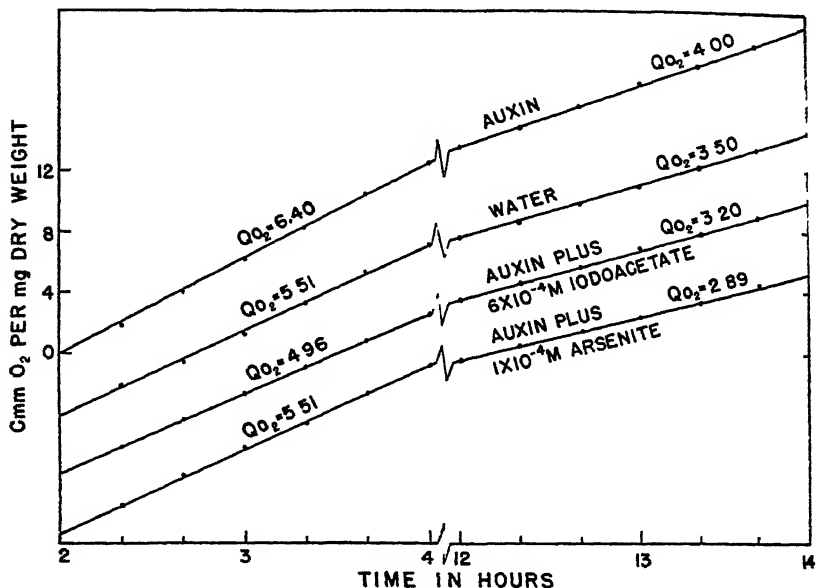


FIG. 1. The rate of oxygen uptake of pea stem sections in solutions. Data typical of all experiments in Table I. Sections cut and put in solutions at time zero; origin of the  $O_2$  uptake coordinates are arbitrary.

auxin. It is seen that the three inhibitors have different effects on the respiration rates of the sections. Iodoacetate depresses the rate of oxygen uptake below that of the water controls during the first period of measurement and the degree of inhibition generally parallels growth inhibition. The rate during the second period remains at the same level as in the water control for all concentrations except the lowest. Arsenite also reverses the stimulation of respiration caused by auxin but, unlike

iodoacetate, the inhibition is slight at first and becomes stronger during the second period. Fluoride, in contrast to the other inhibitors, causes only a very slight inhibition of respiration up to  $10^{-2}$  *M*, and this changes to a distinct stimulation during the second, or nongrowing, period.

TABLE I  
*Respiration Rates of Sections of Etiolated Pea Internodes*

Treatment	Cu. mm. $O_2$ /mg. dry wt./hr. ( $QO_2$ ) <sup>a</sup>		Per cent inhibition of growth <sup>d</sup>
	After 2 hr.	After 12 hr.	
Water control	5.32 <sup>b</sup>	3.34 <sup>b</sup>	—
Auxin 1 mg. l.	6.77 <sup>b</sup>	3.82 <sup>b</sup>	—
Auxin plus:			
$2 \times 10^{-4}$ <i>M</i> iodoacetate	5.13	3.71	
$4 \times 10^{-4}$ <i>M</i> iodoacetate	4.84	3.44	
$6 \times 10^{-4}$ <i>M</i> iodoacetate	4.69 <sup>c</sup>	3.32 <sup>c</sup>	50.0
$8 \times 10^{-4}$ <i>M</i> iodoacetate	4.47	3.31	
$10^{-3}$ <i>M</i> iodoacetate	4.34	3.34	
$5 \times 10^{-5}$ <i>M</i> arsenite	6.24	2.98	
$10^{-4}$ <i>M</i> arsenite	5.51 <sup>c</sup>	2.89 <sup>c</sup>	48.0
$2 \times 10^{-4}$ <i>M</i> arsenite	5.00	2.60	
$4 \times 10^{-4}$ <i>M</i> arsenite	3.37	1.23	
$2 \times 10^{-3}$ <i>M</i> fluoride	6.94	4.58	
$4 \times 10^{-3}$ <i>M</i> fluoride	6.25	4.65	
$5 \times 10^{-3}$ <i>M</i> fluoride	6.18 <sup>c</sup>	4.45 <sup>c</sup>	50.0
$6 \times 10^{-4}$ <i>M</i> fluoride	6.10	4.29	
$10^{-2}$ <i>M</i> fluoride	6.56	4.19	
$10^{-1}$ <i>M</i> fluoride	3.20	0.46	

<sup>a</sup> Each vessel contained five 15-mm. sections in 3.0 ml. of growth solution; 0.2 ml. 20% KOH in well.

<sup>b</sup> Average of 10 determinations. The average increase due to auxin is 27% in the first 2 hr. and 15% after 12 hr.

<sup>c</sup> Average of 6 determinations; all others average of 2 to 4 determinations.

<sup>d</sup> Derived from Figs. 1 and 2 of preceding paper (4).

If we consider the concentrations of the inhibitors which cause a 50% inhibition of growth, the effects in the first period are: iodoacetate ( $6 \times 10^{-4}$  *M*) 31% reduction; arsenite ( $1 \times 10^{-4}$  *M*) 19% reduction; fluoride ( $5 \times 10^{-3}$  *M*) 9% reduction.

*Respiratory Quotients*

Respiratory quotients were determined by Warburg's direct method as described by Umbreit *et al.* (13). The measurements were identical with the  $Q_{O_2}$  determinations except that the gas exchange of two similar samples of tissue was followed in respirometers containing the same growth solutions, one with KOH in the center well and one with water. The R.Q. values obtained at the 20-min. intervals were averaged separately for the two 2-hr. periods, since they showed no observable trend during these periods.

TABLE II  
*Respiratory Quotients of Sections of Etiolated Pea Internodes*

Treatment	Respiratory quotient (R.Q.)	
	After 2 hr.	After 12 hr.
Water control	1.07 <sup>a</sup>	0.98 <sup>a</sup>
Auxin 1 mg./l.	0.98 <sup>a</sup>	0.84 <sup>a</sup>
Auxin plus:		
2 × 10 <sup>-4</sup> M iodoacetate	1.00	0.90
4 × 10 <sup>-4</sup> M iodoacetate	1.02	0.97
6 × 10 <sup>-4</sup> M iodoacetate	1.07 <sup>a</sup>	0.99 <sup>a</sup>
8 × 10 <sup>-4</sup> M iodoacetate	1.10	1.07
5 × 10 <sup>-5</sup> M arsenite	1.04	1.07
10 <sup>-4</sup> M arsenite	1.08 <sup>a</sup>	1.14 <sup>a</sup>
2 × 10 <sup>-4</sup> M arsenite	1.13	1.21
5 × 10 <sup>-3</sup> M fluoride	1.10 <sup>a</sup>	1.07 <sup>a</sup>

<sup>a</sup> Average of 4 determinations; all others the average of 2 determinations.

The R.Q. values obtained in this way are presented in Table II. For sections growing in water, the R.Q. is evidently close to unity; auxin causes a slight decrease in this, which in the second period is accentuated. Iodoacetate prevents the decrease in R.Q. caused by auxin, as do arsenite and fluoride. In the higher inhibitor concentrations, the R.Q. is consistently above unity. Thus in general the effects of auxin and of inhibitors are opposite; auxin tends to lower the R.Q. and the inhibitors tend to raise it.

## ETHER SOLUBLES

*Lipides*

The content of neutral ether-soluble material, the lipides, was determined by extraction with ether from the dried tissue. The sections, after growth, were soaked briefly in a very dilute solution of sodium

TABLE III

*Lipide Content of Pea-Stem Sections before and after Growth*

Treatment	Neutral ether solubles as per cent of initial dry wt. <sup>a</sup>	Change during growth or inhibition
Initial sample	8.95	—
Water control	6.64	-2.31
Auxin 1 mg./l.	6.18	-2.77
Auxin plus:		
$6 \times 10^{-4}$ M iodoacetate <sup>b</sup>	7.44	-1.51
$10^{-4}$ M arsenite <sup>b</sup>	8.60	-0.35
$5 \times 10^{-3}$ M fluoride <sup>b</sup>	9.82	+0.87

<sup>a</sup> Average of 4 determinations using samples of 30 sections each.<sup>b</sup> Concentration giving 50% growth inhibition.

carbonate (ca. 0.01 M) to form salts of any acidic substances and thereby prevent their extraction by ether. The sections were then dried at 105°C. to constant weight, and ground to a fine powder which was transferred to an extraction thimble (Whatman ether extracted, 10 × 50 mm.) and extracted with dry, freshly distilled ether for 24 hr. in a micro-Soxhlet apparatus. The ether was then removed from the extract and the residue dried and weighed. In order to check that this contained

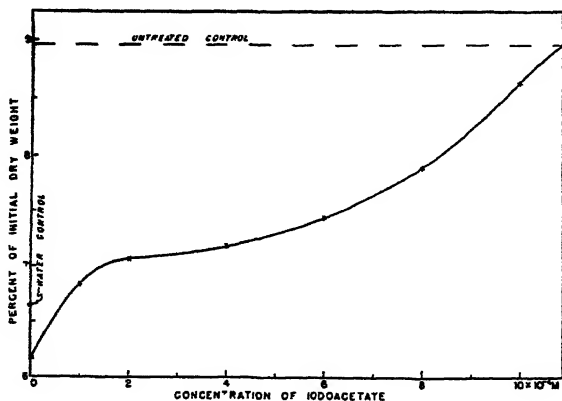


FIG. 2. The effect of iodoacetate on the neutral ether solubles. Pea-stem sections treated 24 hr. in solutions. All concentrations of inhibitor were used in the presence of 1 mg. l. indoleacetic acid. Each point represents the average of 2 or more determinations.



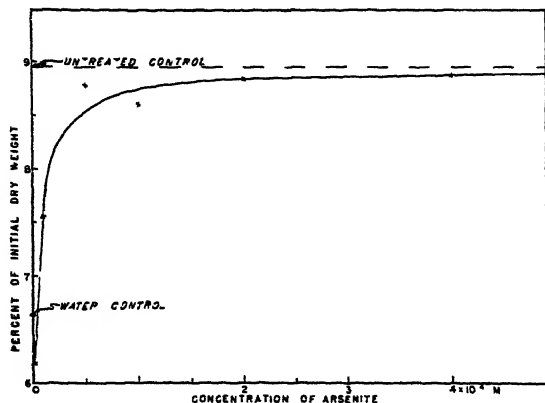


FIG. 3. The effect of arsenite on the neutral ether solubles. Conditions and data similar to Fig. 2.

only neutral material, the combined residues from 12 determinations were extracted with hot water. A negligible amount dissolved.

Table III shows the results of these determinations for the three controls and for the 50% inhibiting concentrations of the three in-

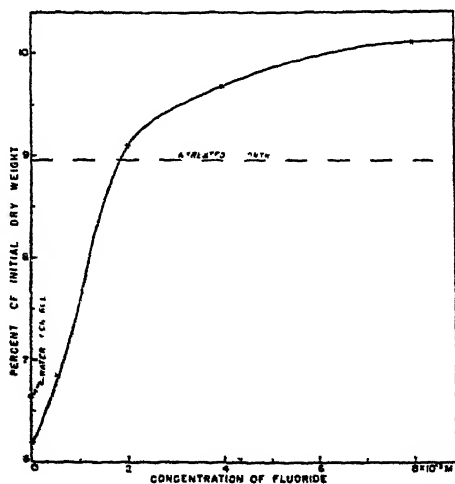


FIG. 4. The effect of fluoride on the neutral ether solubles. Conditions and data similar to Fig. 2.

inhibitors. It is seen that the lipid content decreased by 26% in the water control and by 31% during growth in auxin.

The striking effects of increasing concentrations of the inhibitors on this lipid consumption are shown in Figs. 2, 3, and 4. It is seen that in all cases the consumption of lipid falls off with increasing inhibition. Iodoacetate (Fig. 2) caused a gradual rise in the lipid content, but at the 50% growth inhibition point ( $6 \times 10^{-4} M$ ) it allowed a little more than half the utilization taking place in the controls. Arsenite (Fig. 3) caused a very sharp return to the initial untreated value, which is virtually complete at  $1 \times 10^{-4} M$ , the 50% inhibition point. Lastly, Fig. 4 shows that fluoride not only rapidly reversed the consumption of lipid caused by auxin, but at higher concentrations even brought about an actual net production of lipid. At the 50% inhibition point ( $5 \times 10^{-3} M$ ) the lipid content was raised about 10%.

### *Organic Acids*

In addition to lipides there is a relatively small amount of free organic acids in plant tissue. The determination of these acids is subject to certain difficulties, and results of an extensive series of experiments with both pea stems and coleoptiles will be published elsewhere. For the present purpose the extent of formation or disappearance of total organic acids under the growing conditions was felt to be sufficient.

The total organic acids were therefore determined by acidification, extraction with ether, and titration from pH 8 to pH 2.6 (8)<sup>2</sup>. The resulting content in microequivalents was converted to weights by using the equivalent weight of malic acid, *i.e.*, 67. This procedure is fully justified by the fact that the equivalent weights of most of the other plant acids are very close to this figure.

It was found that the organic acids of the untreated stem sections totalled 6.2% of the dry weight. After 24 hr. in water this fell to 5.0% and after 24 hr. in auxin to 4.7%. There is thus a decrease of organic acids during maintenance or minimal growth and a slight further decrease when growth in auxin occurs. When the three inhibitors were added at the levels giving 50% growth inhibition, the disappearance of total organic acids was little changed. The percentages of initial dry weight of organic acids after 24 hr. in auxin plus the inhibitors were:

<sup>2</sup> These determinations were made by Dr. W. D. Bonner, Jr., to whom the authors are greatly indebted.

iodoacetate 4.7, arsenite 4.4, and fluoride 4.6. It appears, however, that the individual acids may show marked changes during inhibition, the disappearance of malate and pyruvate being inhibited by arsenite (9). Nevertheless, these effects have little influence, within the experimental error, on the figures for total acids.

### DISCUSSION

The rates of oxygen uptake, presented in Table I, show very little correlation with the amounts of sugar consumed [see the previous paper (4)]. The auxin controls respire at a considerably greater rate than the water controls but use the same amount of reducing sugar and only slightly more sucrose. The inhibited sections consume half again as much sugar as the auxin controls, yet they take up either practically the same amount of oxygen (fluoride-inhibited) or from 20 to 30% less (iodoacetate- and arsenite-inhibited). Thus the additional sugar which disappears when growth is inhibited is evidently not respired.

The clear increase of  $Q_{O_2}$  by auxin in this tissue is noteworthy; it occurred in every experiment and was quantitatively reproducible. In contrast, *Avena* coleoptile sections have been found by most workers to show no such increase [for earlier literature see (2, 9)], but recently J. Bonner (3) reported an increase of 20–25% with indoleacetic acid (10 mg. l. at pH 4.5) and Kelly and Avery (5) a similar increase with relatively high concentrations of 2,4-D at pH 7.

When we turn to the respiratory quotients, however, it can be seen that they interrelate closely with the determinations of fats and organic acids.

A small but definite decrease in organic acids is seen in all cases. The oxidation of these substances, with R.Q. > 1 (1.33 for malic and citric acids), raises the observed R.Q. slightly, and hence without them the R.Q. for the respiration of other substrates would be very close to 1 for sections in water. For sections in auxin it falls toward the value for fats, i.e., auxin promotes fat oxidation. The increased  $Q_{O_2}$  in auxin with very little accompanying decrease in sugars [cf. preceding paper (4)] must thus be due, at least in part, to accelerated oxidation of fats. This may, of course, be complete or partial oxidation; the relatively large decrease in R.Q. for a small increase in fat consumption would suggest the latter. The three inhibitors again raise the R.Q.; in other words they inhibit fat oxidation, which is what Figs. 2, 3, and 4 demonstrate

directly. The data are thus completely consistent, assuming that the neutral ether solubles consist principally of fats.

Iodoacetate clearly exerts less marked inhibition on fat consumption than do the other two. It is believed, however, that its effect is fundamentally the same, for the following reason. Sections in iodoacetate plus auxin excrete a considerable fraction of their soluble contents (about 15% of the dry weight, at the 50% inhibiting concentration of iodoacetate) into the solution. This fact has been reported briefly (10) and will be taken up in detail in a later paper. For our present purposes it will suffice to note that an inhibition of fat oxidation, coupled with some excretion of the partly oxidized metabolites, would account for the partial return of the lipide values in Fig. 2 to the initial value. In an earlier paper (11) reasons have been given for concluding that iodoacetate and arsenite inhibit growth through interference with the same mercapto-enzyme system, in spite of apparent differences between the behavior of these two inhibitors. The present data do not conflict with this view.

These experiments have bearing on earlier work by Albaum and Eichel (1). In their study it was observed that during the first 72 hr. of growth of intact etiolated *Avena* seedlings, total ether solubles (which according to their methods would include organic acids as well as fats) are consumed rapidly, while the level of reducing sugar remains essentially constant. The R.Q. during this time is near 0.8 and growth is not strongly inhibited by iodoacetate ( $2 \times 10^{-5}$  M) in contact with the roots. Only later does the R.Q. approach 1 and the growth become iodoacetate-sensitive. On the basis of these observations, Albaum and Eichel conclude: (a) that fat is the chief metabolite during early growth (the sole metabolite up to 48 hr.) and that sugars are immune to destruction during this time, (b) that fat metabolism is insensitive to iodoacetate, which must inhibit growth by blocking glycolysis at the triose phosphate stage, and (c) that during growth there is a gradual transition from fat destruction to sugar destruction.

To some extent our observations on pea stems are in agreement with these findings with coleoptiles; that is, they show that both organic acids and fats are consumed during growth. But their interpretation of these facts may be open to question. A much more likely interpretation, for which considerable evidence exists, is that sugars as well as fats and organic acids are being oxidized during early growth as well as later, but that the level of reducing sugar is maintained approximately constant by conversion of fats to sugar. It is realized that such a mechanism—conversion of fats to sugar and subsequent oxidation of sugar—is equivalent to simple oxidation of fats. However, this mechanism does

not require the total stagnation of the sugar oxidizing system as is implied by Albaum and Eichel. Furthermore, good evidence that this system is operating in germinating plants is offered by Mapson *et al.* (6) who showed, in a metabolic study of cress seedlings, that fat and sucrose decrease rapidly during growth, while the reducing sugars increase by considerably more than the decrease in sucrose. These data were interpreted as indicating the conversion of fat reserves to sugar and the oxidation of some of this sugar.

Further evidence of this mechanism of fat consumption is found in the earlier work of Murlin *et al.* (7) with castor bean seedlings. Here fat is the principal seed reserve material and is very rapidly converted to sugar, some of which is oxidized but much of which appears as polysaccharides in the cell walls of the young plant. The over-all R.Q. of these seedlings is as low as 0.3.

The R.Q. of 0.8, observed by Albaum and Eichel, would be expected as the resultant of a combination of organic acid metabolism (R.Q.  $> 1$ ), sugar metabolism (R.Q.  $= 1$ ) and fat metabolism (R.Q.  $< 1$ ), plus some conversion of fat to sugar (R.Q. near zero.) Because of the fact that organic acids are known to be consumed during growth (9), the over-all R.Q., including the oxidation of some carbohydrate, would be expected to be higher than 0.8. Hence it is probable that some process of very low R.Q. such as conversion of fat to sugar does participate.

Albaum and Eichel's statement that fat metabolism is insensitive to iodoacetate is based on the very tenuous evidence that early growth is insensitive to this inhibitor. In fact, the present experiments show fat consumption to be considerably more sensitive to iodoacetate than is sugar consumption. The insensitivity of young coleoptiles to iodoacetate is almost certainly due to their high content of organic acids (10) which protect against iodoacetate inhibition (12).

The third conclusion, that a transition from fat to sugar metabolism takes place on aging, is an unnecessary complication. The much simpler view, that sugar metabolism is continuous and that the conversion of fats to sugar slows down simply because most of the fat is consumed, is borne out by all the experimental evidence.

In conclusion it is clear that auxin and the inhibitors influence many aspects of the metabolism of pea-stem tissue. Whether these changes all follow from the catalysis of a single process, or the inhibition of one or more single processes, can not be determined as yet. It is evidently of little value to attempt to consider any single aspect of metabolism by itself when the whole picture is so interrelated. For this reason analyses of other constituents have been made and will be reported subsequently.

#### SUMMARY

Indoleacetic acid stimulates the respiration of pea-stem sections by 15 to 25% over that of sections grown in water alone. There is an accompanying decrease of R.Q. from 1.0 to about 0.9.

Iodoacetate and arsenite at 50% growth-inhibiting concentrations reverse this stimulation of  $Q_{O_2}$  and raise the R.Q. to 1.1.

Fluoride increases the R.Q. by the same amount but does not appreciably inhibit oxygen uptake up to  $10^{-2}$  M.

The fat content of the sections decreases by about 25% in water and about 30% in auxin. The three inhibitors prevent this utilization of fat. Indeed, in the presence of fluoride the fat content actually increases. It appears that the consumption of fat has a close relationship to growth.

Total organic acids decrease by about 20% in water and by about 25% in auxin. The three inhibitors have no appreciable effect on total organic acid utilization.

It is concluded that fats are continuously being converted to sugars and that this conversion is prevented by the three inhibitors. This would explain the lowering of the reducing sugar content, raising of the fat content, small decrease in oxygen uptake and rise of the R.Q.,—effects caused by all three inhibitors. The relationships between carbohydrate and fat metabolism in these and previous experiments are discussed, particularly in regard to their bearing on growth.

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## The Determination of Esterase in Animal Tissue Homogenates<sup>1</sup>

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### INTRODUCTION

The majority of the methods designed to determine lipase or esterase activity are based on the titration of acid liberated from a chosen substrate or involve the measurement of the change in surface tension resulting from hydrolysis (1). Rona and Lasnitski (2) developed a simplified method for determination of serum esterase by measuring carbon dioxide evolved from bicarbonate by the products of hydrolysis of tributyrin. Singer and Hofstee (3) made use of a similar manometric method for the study of wheat-germ lipase, using a variety of substrates including Tween 20 and Tween 40, poly-oxy-olefin derivatives of lauric and palmitic acid esters of sorbitan. These compounds, as pointed out by Gomori (4) and Archibald (5), are convenient substrates for measurement of lipase activity, since, due to their solubility in water, they provide a homogeneous system. Simple esters of lauric and palmitic acids can be used only in an emulsified state.

Since we were seeking a simple method which could be used to determine "lipase" activity of small samples of animal tissue, we undertook the development of a method similar to that of Singer and Hofstee (3) for the study of tissue homogenates. Tween 20 was chosen for our study since Archibald (5) had provided information on the kinetics of the action of pancreatic lipase on this substrate.

<sup>1</sup> This investigation was supported in part by a grant from the Committee on Research in Endocrinology, National Research Council, and in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

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## EXPERIMENTAL

The chief sources of the enzymes used throughout this study were homogenates of rat liver and kidney, although some study of other tissues was also made. Tissues from at least three animals were pooled in each determination in all experiments except where otherwise indicated. Values reported herein are for the studies on liver only, but most of the data on enzyme-substrate kinetics are equally applicable to kidney tissue.

All animals used in the development of the method were adult males of the Holtzman strain, weighing 300-500 g. The tissues were removed immediately after the animals were killed and just before the beginning of each experiment. Tissues were homogenized in sharp-pointed ground glass tubes with a small quantity of water, and were diluted to concentrations of 1% by volume.

The reactions were carried out in single side-arm Warburg flasks in an atmosphere of 95% nitrogen: 5% carbon dioxide at 38°C. except where otherwise specified. The system consisted only of the buffered substrate, sodium bicarbonate, and the tissue homogenate in concentrations indicated below. Measurements of carbon dioxide evolved were made at the ends of a number of consecutive 10-min. periods. All values are the means of values for at least five consecutive 10-min. periods.

*Effect of pH on Enzyme Activity*

Figure 1 shows the results of variation in pH on the activity of liver esterase. The Tween concentration used in these experiments was 25% by volume; the bicarbonate concentration was varied in relation to the pH, temperature, and carbon dioxide concentration (6). The system also contained other buffers of the proper pH. Since no single ordinary buffer covers this pH range (7.5-8.8) effectively, it was necessary to use a combination of buffers: in addition to the carbon dioxide-bicarbonate buffer, phosphate and barbital buffers in final molarities of .0165 were used.

From the data in Fig. 1 it is apparent that the velocity of hydrolysis of this substrate by the liver enzyme is greatest at pH 8.4. The irregularity of the points near the optimum pH is probably due to the use of homogenate as an enzyme source rather than using a purified preparation, since under the conditions of this experiment the enzyme concentration was found to vary slightly from one determination to another because of animal variation.

*Effect of Substrate Concentration on Activity*

The effect of substrate concentration on reaction velocity is shown in Fig. 2. The concentration range in which the substrate is in excess



for the quantity of enzyme used here is that between 25% and 40% Tween. The Michaelis constant  $K_s$  (7) for the system was calculated from these data to be 2.6%.\*

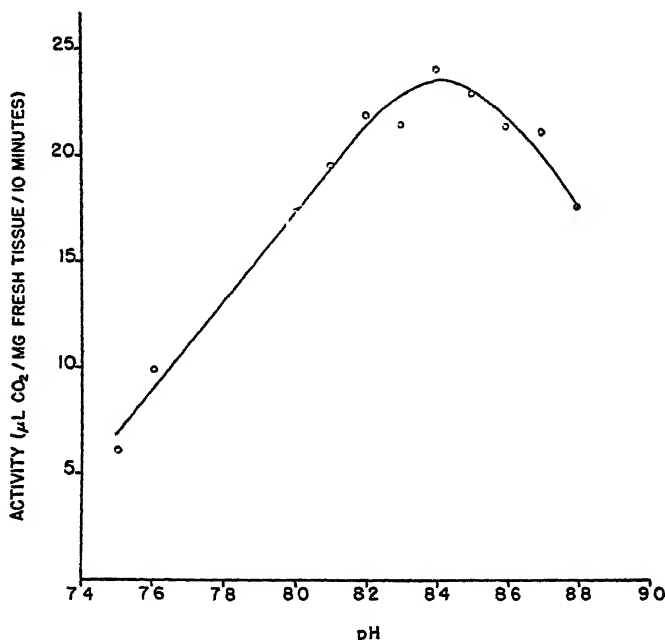


FIG. 1. Effect of pH on the activity of rat liver esterase. Each point represents values obtained on one to six determinations.

#### *Relation Between Enzyme Concentration and Activity*

The measured activity is proportional to the quantity of homogenate used through a limited range as indicated by Fig. 3. In liver tissue concentrations higher than 3 mg./flask, one frequently obtains apparent inhibition of activity. A large portion of this inhibition is probably due to retention of carbon dioxide in the system since a high rate of activity will cause a marked increase in the carbon dioxide concentration of the atmosphere.

\* This is the value if one eliminates the values obtained at substrate concentrations of 1% and 20% (see Fig. 2) as being grossly in error; if all points are included, the  $K_s$  is closer to 3.1%.

*Relation Between Incubation Time and Activity*

Under optimal conditions the reaction velocity remained essentially constant throughout a 90-min. period.

*Effect of Temperature on Enzyme Activity*

Experiments were performed with tissue concentration, substrate concentration, and pH at optimal levels, but with varied temperature.

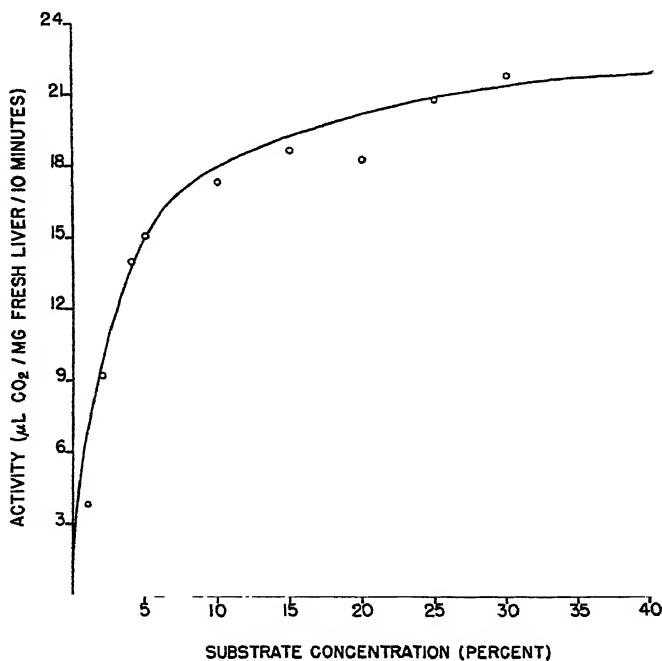


FIG. 2. Effect of substrate concentration on the activity of rat liver esterase. Determinations carried out at pH 8.4, 38°C., with 2.0 mg. fresh tissue. Each point represents values obtained from two to four determinations.

Between 30–44°C. there is a straight-line relationship between temperature and reaction rate ( $Q_{10}$  2.7). It was impractical to carry the water-bath temperature beyond 44°C.; the optimum temperature was not, therefore, determined.

*Miscellaneous Experiments to Establish Validity of Method*

An experiment was performed with tissue heated to 80°C. for 5 min. This treatment completely inactivated the enzyme. No carbon dioxide evolution beyond that of a tissue-free control was obtained. Incubation of unheated tissue in the absence of the substrate produced no significant carbon dioxide evolution.

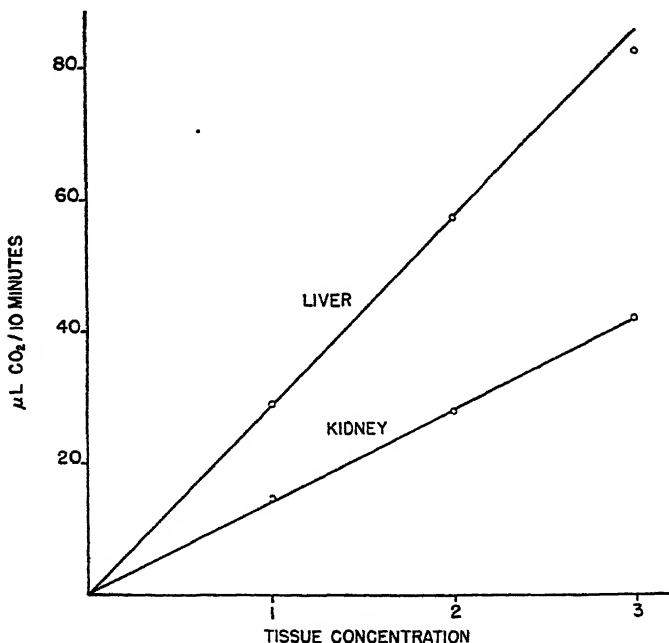


FIG. 3. Effect of tissue concentration on the activity of liver and kidney esterase. Tissue concentration is expressed as milligrams fresh tissue/flask. Determinations carried out with 25% Tween, pH 8.4, 38°C.

In order to determine any possible effect of the anaerobic conditions used, experiments were performed at an atmosphere of 95% oxygen: 5% carbon dioxide. No significant difference in activity was noted.

To determine whether the carbon dioxide was actually being evolved through the action of enzymatically liberated fatty acid,<sup>4</sup> a large

<sup>4</sup> According to information kindly supplied by the Atlas Powder Company, most of the fatty acid in Tween 20 is lauric but there are minor proportions of capric and myristic acids incorporated into the product as well.

amount of liver homogenate (5 mg.) was used in each of 10 flasks and the enzyme was allowed to act for 90 min. An equivalent amount of reaction mixture, lacking the tissue homogenate, was incubated similarly. At the end of the incubation period the combined flask contents and the control, to which a corresponding amount of tissue was added just before acidification, were treated with  $\text{NaH}_2\text{PO}_4$  and extracted with Archibald's ether-petroleum ether mixture (5), and the extracts were titrated with standard alkali. The extract, which had been incubated with the enzyme, contained 0.78 mequiv. more acid than the control. It was determined that the extract contained acids of the lauric acid series, using the qualitative test of Ludwig and Haupt (8).

### *Routine Method for Esterase Estimation*

The experiments described above made possible the definition of conditions for routine analysis of tissue esterase. The method now being used in this laboratory is described in detail below.

### *Solutions*

a. *Buffered Substrate.* Dissolve 8.955 g.  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  and 5.154 g. sodium diethylbarbiturate in 500 ml. glass-distilled water, and adjust to pH 8.4 with 10% phosphoric acid. Mix 4 parts of this buffer with 3 parts of Tween 20 and 1 part water and allow the mixture to stand overnight in the refrigerator. Adjust the mixture to pH 8.4 with dilute phosphoric acid and/or sodium hydroxide. This solution may be used for 3 days, but it is advised that the pH be carefully checked before each determination.

The adjustment of the pH should be done slowly and as accurately as possible (a Beckman pH meter was used in our study) since the equilibrium between carbon dioxide and bicarbonate depends on this procedure.

b. *Sodium Bicarbonate, 0.71 M.* This solution should be made up fresh at least every 4 days.

### *Procedure*

Two ml. of the buffered substrate solution, 0.6 ml. of 0.71 M sodium bicarbonate, and sufficient water so that when the homogenate is added the total volume will be 3.0 ml. are placed in the flask compartment of single side-arm Warburg flasks. This results in a concentration of 25% substrate. Note from Fig. 2 that this is barely within the optimum range for 2.0 mg. of fresh rat-liver homogenate. Higher concentrations of Tween may be used, but the material is so viscous that handling solutions of greater concentration than this is extremely difficult, and bringing such a sirupy mixture to the proper pH is very tedious. For work with ordinary concentrations of normal rat tissues this substrate concentration is sufficient to saturate the enzyme.

Homogenate (0.05–0.20 ml. of a 1% homogenate in the case of rat liver) is placed in the side-arm. The flasks are attached to the manometer and gassed from a manifold with 95% nitrogen: 5% carbon dioxide for 10 min. Following this, the flasks are transferred to the Warburg bath and allowed to equilibrate for 5 min. with shaking. The homogenate is then rinsed into the main portion of the flask. The homogenate and substrate are thoroughly mixed by a 15-min. shaking. At the end of this period the manometer fluid columns are adjusted and an initial reading is made. The subsequent carbon dioxide evolution is recorded at 10-min. intervals for the desired length of time. The usual thermobarometer is used, and one flask containing all components of the system except the tissue is used as a control. Values from tissue-containing flasks are corrected for any small carbon dioxide evolution or uptake from this control flask. All determinations are made in duplicate.

We have elected to express results as microliters of carbon dioxide evolved/10-min./mg. fresh tissue, taking the mean evolution of at least 5 consecutive periods.

### *Survey of Some Rat Tissues*

Of the tissues tested for esterase activity (lung, kidney, brain, testis, liver, pancreas, corpus luteum, adrenal gland, and placenta) the liver and kidney had by far the greatest activity, liver being about twice as

TABLE I  
*Esterase Activity of Livers of Rats of Both Sexes in Three Age Groups*

Age in days	No. of rats	Sex	Liver esterase ( $\mu$ l. CO <sub>2</sub> /10 min./mg. fresh tissue)
19	2	Male	4.0
	2	Female	3.6
90	2	Male	27.9
	2	Female	10.2
180	2	Male	23.4
	2	Female	12.5

active as kidney, weight for weight. The brain, pancreas, and placenta were completely lacking in activity. The activity of testis was about one-third that of the liver, and lung had about one-sixth the activity of liver. Small but measurable activity was found in the corpus luteum and adrenal gland.

Table I shows values obtained on livers of rats of both sexes in three age groups. Experiments designed to cast more light on the difference between the activity of male and female rat liver are now in progress.

## DISCUSSION

It is believed that the method described here has value as a tool in laboratories interested in the significance of variation of enzyme systems in tissues under varying physiological conditions. It is quite sensitive since activity can be reliably measured with as little as 0.5 mg. of fresh rat liver. In comparison with other manometric methods for measuring tissue metabolism with homogenates, it is extremely simple so that an untrained operator can become proficient in a short time.

There is some question as to whether the method described is measuring esterase or lipase. Archibald (5) adequately demonstrated that pancreatic lipase hydrolyzed Tween 20 optimally at pH 6.8, 23–28°C., at substrate concentrations greater than 37%. All of these conditions are far from the optima we have established for the liver enzyme.

Weinstein and Wynne (9) studied lipase in an extract of pancreas and found activation by potassium cyanide, inhibition by aldehydes and fluoride. The enzyme measured in this study is inhibited up to 65% by 0.01 *M* fluoride, but a similar concentration of benzaldehyde does not inhibit, nor does cyanide activate or inhibit the system.

Bloor (10) distinguishes between lipases and esterases chiefly on the basis that esterases work best on esters of the lower fatty acids, becoming less effective as the fatty acid chain lengthens, and that lipases are most active on the esters of the longer-chain fatty acids. Since the enzyme concerned in this study works at a high velocity on an ester of a long-chain fatty acid ( $C_{12}$  acid), the enzyme could be called a lipase.

In view of the confused status of the literature on the distinction between esterases and lipases, however, it is felt that the nomenclature is not of great importance. We have chosen to call this enzyme "tissue esterase" because it hydrolyzes an ester bond, it does not have the same behavior toward activators and inhibitors as does pancreatic lipase, and it is found in highest concentration in the liver, which also has the greatest "esterase" activity as measured with other methods by other workers (4, 11, 12).

## SUMMARY

A method is described for the estimation of esterase activity in animal tissues. The substrate is Tween 20, and the technique involves measurement of carbon dioxide liberated from bicarbonate by the acid produced by action of the enzyme.

The kinetics of the hydrolysis of Tween 20 by the esterase of rat liver have been investigated. The optimum pH is 8.4. Reaction velocity is proportional to enzyme concentration in the presence of excess substrate and remains constant for over an hour. The Michaelis constant is 2.6%. The velocity increases more than threefold between 30°C. and 44°C.

The enzyme is found in measurable quantity in liver, kidney, lung, testis, adrenal gland, and corpus luteum. It is absent in brain, pancreas, and placenta.

Adult male rat liver has two to three times the esterase activity of adult female liver. There is no significant difference in activity in different sexes in infantile animals; the activity of livers of infantile animals is much lower than that of adults.

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# Inhibitory Effect of Thiocyanate upon Oxidations Mediated by Liver and Kidney<sup>1</sup>

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## INTRODUCTION

It is well-known that the cyanide ion inhibits certain enzymatic reactions (1). However, the effect of the thiocyanate ion in enzymatic inhibition has received little attention. Thiocyanate inhibits the action of carbonic anhydrase (2) and catalase (3). This ion also acts as a goitrogen (4) and a hypotensive agent (5). Since thiocyanate has been used therapeutically to produce a lowering of blood pressure in some patients, and little is known of its mechanism, it was decided to study its effect on tissue *in vitro*.

In the present investigation the effect of various levels of the thiocyanate ion on oxygen uptake was studied in broken-cell preparations of organs of guinea pigs with and without substrates. In order to obtain a degree of inhibition comparable with that produced by cyanide, much higher concentrations of thiocyanate were found necessary when no substrate was present. Oxidation of amino acid substrates was strongly inhibited at low concentrations of thiocyanate, whereas the oxidation of the corresponding amines was not affected. Similar low concentrations of cyanide did not inhibit the oxidation of amino acids and amines.

## EXPERIMENTAL

Liver, kidney, brain, heart, lung, and spleen of guinea pigs were blended briefly with 1 ml. of 0.05 *M* potassium phosphate, pH 7.80, per gram of wet tissue and then squeezed through muslin.

The rate of oxygen uptake of 1.0 ml.-aliquots of the tissue preparation to which had

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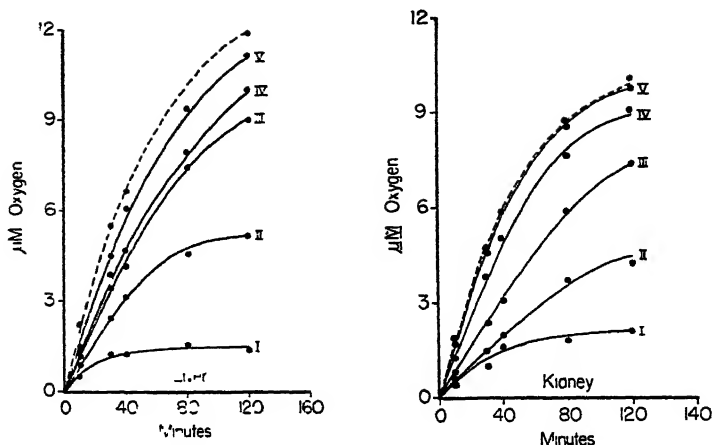


FIG. 1. Effect of varying concentrations of thiocyanate upon oxygen uptake of liver and kidney. The broken lines represent the oxygen uptake of the tissue in the absence of thiocyanate or presence of 0.1 *M* sodium or potassium chloride. Curves I, II, III, IV, and V represent final concentrations of 1, 0.1, 0.01, 0.001, and 0.0001 *M* thiocyanate, respectively.

been added 1.0 ml. of buffer containing substrate, inhibitors, or both, was measured at 37.5°C. with the usual Warburg apparatus; the pH of the final mixture was 7.4. The difference between the oxygen uptake in the presence of substrate and that of its suitable control was considered a measure of the oxidation of the substrate.

The data in Fig. 1 show the effect of increasing concentrations of thiocyanate on the rate of oxygen consumption of liver and kidney. In tissues, 1 *M* thiocyanate completely inhibited the oxygen uptake while 0.1 *M* thiocyanate usually produced an inhibition of 50%. To obviate the possibility of chance impurities inhibiting the rate of oxidation, three commercial preparations of potassium thiocyanate and one of

TABLE I  
*Comparison of Inhibition of Oxygen Uptake of Homogenates of Various Tissues by Thiocyanate and Cyanide*

Values are expressed at  $\mu$ moles oxygen uptake in 120 min.

Inhibitor	Liver	Kidney	Brain	Heart	Spleen	Lung
0	13.5	12.8	18.7	7.1	5.1	4.2
0.001 <i>M</i> CN <sup>-</sup>	7.6	8.1	8.3	1.8	1.6	2.1
0.1 <i>M</i> CNS <sup>-</sup>	6.8	6.0	7.4	1.8	1.7	2.0
0.001 <i>M</i> CN <sup>-</sup> plus 0.1 <i>M</i> CNS <sup>-</sup>	6.4	5.7	7.0	1.7	1.5	2.0

sodium thiocyanate were used. All preparations of both the potassium and sodium salt gave identical results. As a further precaution, equimolar solutions of potassium and sodium chloride were tested and produced no significant inhibition.

A cyanide-like inhibition of oxygen uptake of the various tissue preparations without substrate was produced by thiocyanate, although high concentrations were required. (See Table I.) In all of the organs studied 0.1 *M* thiocyanate showed an inhibition of at least 0.5; comparable inhibition was obtained with 0.001 *M* cyanide. When these amounts of both cyanide and thiocyanate were added to the tissue preparation, no further inhibitory effect was noted.

TABLE II

*Inhibitory Effect of Thiocyanate Upon Oxidations Effected by Liver*  
One ml. of liver homogenate and 1.0 ml. of phosphate buffer pH 7.80  
containing thiocyanate, amino acid or amine.

Substrate	Rate of oxidation $\mu\text{moles O}_2 /$ 120 min.	Concentration of CNS <sup>-</sup> producing inhibition of 0.5
0.02 <i>M</i>		<i>M</i> $\times 10^{-4}$
L-Phenylalanine	8.2	3
DL-Phenylalanine	9.3	10
Phenylethylamine	16.2	>1000
L-Tyrosine	5.8	6
DL-Tyrosine	6.2	11
Tyramine	18.7	>1000
L-Leucine	12.1	8
DL-Leucine	11.9	14
Isoamylamine	20.0	>1000
DL-Tryptophan	7.9	15
Tryptamine	15.6	>1000
L-Histidine	7.1	18
Histamine	10.1	135
DL-Alanine	11.5	20

The data in Table II indicate that low concentrations of thiocyanate ion inhibited the rate of oxidation of L- and DL-phenylalanine, L- and DL-tyrosine, L- and DL-leucine, DL-tryptophan, L-histidine, and DL-alanine by liver preparations. The rate of oxidation of the amines corresponding to these amino acids, however, with the exception of histamine, was not altered even in the presence of a high concentration (0.01 *M*) of thiocyanate. Similar results were obtained using homogenates of kidney tissue. Parallel experiments on cyanide inhibition showed that concentrations below 0.001 *M* cyanide produced no change in either amino acid or amine oxidation.

The degree of inhibition produced by thiocyanate in the case of each substrate alone did not depend upon the sequence in which substrate and inhibitor were added to the tissue. To test whether the inhibition was of the competitive type, experiments were carried out with varying concentrations of DL-alanine and thiocyanate. On application of the Michaelis-Menten equation (6), the ratio of the dissociation constants was not found to be a constant.

## DISCUSSION

It has been generally assumed that the thiocyanate ion does not readily permeate normal cell membranes; in fact, the distribution of the thiocyanate ion in the body has been used as a method for the determination of extracellular fluid volume (7). Wood and Williams (8) have recently shown that the protein of the thyroid gland fixes thiocyanate sulfur to a greater extent than the protein of other tissues; thiocyanate was also shown to penetrate the cells of the adrenal gland and liver in small amounts. They postulated that this ion is a physiologic antagonist to the iodide ion and may form a protein-thiocyanate complex which might block further enzymatic reaction. Davenport (2), in studying the inhibition of carbonic anhydrase, has postulated that thiocyanate combines with the zinc in the enzyme, analogous to the combination of cyanide and iron in the cytochrome system. Jung (3) found that thiocyanate forms a complex with methemoglobin although the combination is less stable than the cyanide, mercapto, and azide complexes.

Thiocyanate has been reported to lower the blood pressure in some patients with essential hypertension if a blood level of  $1.7 \times 10^{-3} M$  (10 mg.- $\%$ ) is maintained (9). *In vitro*, a level of 0.01  $M$  thiocyanate produces a 50% inhibition of tissue oxidation without substrate. This is in agreement with the work of Friend and Robinson (10). However, at levels of  $5 \times 10^{-4} M$ , thiocyanate produces a 50% inhibition in the oxygen uptake when amino acids are added. Thus, the inhibitory effect of thiocyanate on amino acid oxidation is separate from the general toxic action of this ion and is demonstrable in much lower concentrations than is the latter. Holtz has presented evidence which indicates that hypertension may be involved with an aberration in amino acid metabolism (11). It has been suggested that faulty amino acid metabolism, with impaired ability to deaminate pressor amines, might be a factor in the pathogenesis of hypertension. In view of this hypothesis, it is interesting that thiocyanate has the ability of greatly reducing amino acid oxidation *in vitro* and also frequently lowering blood pressure when given to hypertensive patients. This hypotensive effect is obtained with a blood concentration five times as great as that required to demonstrate inhibition of amino acid oxidation *in vitro*. If we assume that the thiocyanate ion permeates cell membranes only to a small extent, a gradient of this ion would be established between cells and the extracellular fluid. In these experiments, tissue homogenates with

broken cells have been studied, while in studies in human hypertension, the extracellular fluid level has been measured with intact cell membranes. However, in some exploratory experiments,  $1.2 \times 10^{-3}$  *M* thiocyanate produced an inhibition of 0.5 when added to tissue slices (liver) with added L-leucine.

Other evidence has shown that amines may be important in hypertension (12). The present investigation indicates that thiocyanate exerts no effect on the oxidation of the amines studied with the exception of histamine. In this case a specific histaminase has been recognized (13) which is probably affected by thiocyanate.

Therefore, it would appear that thiocyanate exerts at least two separate and distinct actions. The first is a cyanide-like inhibition of the uptake of oxygen by tissue. The broken-cell preparations used required large and quite unphysiological amounts of thiocyanate to produce an inhibition that might be compared to a cyanide action. This effect of thiocyanate at concentrations of 0.1 *M* might possibly block the same respiratory enzyme systems as cyanide (14). The second action of thiocyanate is its effect in low concentrations on the oxygen uptake of added amino acid substrates. In concentrations of about 0.0005 *M*, thiocyanate produces an inhibition of 0.5 on amino acid oxidation. In contradistinction, at similar concentrations cyanide has no observable action on the same substrates. The oxidation of amines was not impaired at these low concentrations of either thiocyanate or cyanide but required very much larger amounts of both of these inhibitors to produce an effect. The possibility of inhibition of decarboxylases, which might be present in the tissue preparation, appeared unlikely in view of the fact that carbon dioxide production was only slightly affected. Inhibition of L-amino acid oxidation was effected at lower concentrations of thiocyanate than those required for DL-amino acids, but due to the presence of D-amino acid oxidase no definite conclusions could be drawn as to which oxidase was primarily affected.

#### ACKNOWLEDGMENTS

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#### CONCLUSIONS AND SUMMARY

The inhibition of the oxygen uptake of broken-cell preparations of organs of guinea pigs has been studied at various concentrations of

thiocyanate. A cyanide-like inhibition of 0.5 is produced at a level of 0.1 *M* thiocyanate. At concentrations of about 0.0001 *M*, however, thiocyanate produces an inhibition of 0.5 of the rate of oxidation of phenylalanine, tyrosine, tryptophan, leucine, and histidine. Under the same conditions the oxidation of phenylethylamine, tyramine, tryptamine, and isoamylamine are not affected. Since the concentration of thiocyanate necessary to inhibit the rate of oxidation of amino acids is less than that required to produce a therapeutic effect in hypertension, thiocyanate could possibly play a physiologic role in lowering blood pressure through the inhibition of amino acid oxidation.

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# The Metabolism of the Appendix (Rabbit) <sup>1</sup>

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## INTRODUCTION

The lymphatic tissue, so important for the defense of the body against aggression by many injurious agents, has not been studied with regard to its metabolic activities. In fact, all investigations in this field have been confined to the measurement of the oxygen uptake or glycolysis: experiments on lymph nodes (1, 2, 3) on hyperplastic tonsils and the thymus (4); and on their main cellular element, the lymphocyte (5-10). The reports on the metabolism of the lymphocytes are, moreover, open to criticism, since they were performed under conditions where cell damage occurred either before or during the experiments. From the old histological observations of Renaut (11) and the recent studies of DeBruyn (12) it is known that the appendix of the rabbit is the most suitable organ for the study of lymphatic tissue, since practically the entire organ is made up of homogeneously distributed lymphatic nodules, the small lymphocytes being in greatest number. The size of the appendix also allows the performance of reliable experiments.

The possible role of the lymphatic tissue in protein synthesis, especially the synthesis of antibodies, and the frequent occurrence of neoplastic growth in this tissue made necessary a study of its metabolic activities. The appendix of the rabbit was selected for this purpose.

## EXPERIMENTAL

Normal adult white rabbits were killed by decapitation, and the appendix removed. It was opened and cleaned under a spray of cold water. The serosa was sectioned

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<sup>2</sup> Atomic Energy Commission Postdoctoral Research Fellow in the Medical Sciences of the National Research Council.

away and the remaining lymphatic tissue, cut with a thin razor blade into sections about 0.4 mm. thick, was placed in ice cold Ringer-phosphate solution. Approximately 90 min. elapsed from the time of the death of the animal to the beginning of the experiments. All the experiments were performed in Warburg vessels and Warburg-Barcroft manometers at 38°. The buffers used were either Ringer-phosphate, pH 7.4 or Ringer-bicarbonate at the same pH, the first saturated with O<sub>2</sub>, the second with O<sub>2</sub>:CO<sub>2</sub> or N<sub>2</sub>:CO<sub>2</sub> (ratio 95:5). For the computation of tissue dry weights, an aliquot of the fluid was taken and precipitated with an equal volume of 10% trichloroacetic acid in a small weighed test tube. After centrifugation the supernatant fluid was discarded and the precipitated protein washed and dried overnight at 100°. This weight was added to the dry weight of the tissue. Respiratory quotients were determined by the method of Warburg and Yabusoe (13); lactic acid, by the method of Miller and Muntz (14); volatile fatty acids, by the method of Friedemann (15); pyruvic acid, by the method of Friedemann and Haugen (16); citric acid, by the method of Speck *et al.* (17); ammonia, by the method of Conway (18); succinic dehydrogenase, by the ferricyanide manometric technique of Quastel and Wheatley (19); glutamate and aspartate, by the method of Cohen (20); aconitase, according to Johnson (21). All substrates were used at a concentration of 0.01 *M* unless otherwise indicated.

### *Respiration of the Appendix*

Possibly because the serosa of the appendix was eliminated, the tissue suspended in the buffer solutions lost from 25–50% of protein which was found in the fluid. However, the respiratory rate remained constant for periods lasting 90–120 min. The average *Q*<sub>O<sub>2</sub></sub> in Ringer-phosphate (cu. mm./mg. dry weight/hr.) from 60 separate determinations was 8.05 ± 0.11, the lowest value being 6.04, and the highest 9.47. This value compares well with other values for different lymphatic

TABLE I  
*The Respiration of Lymphatic Tissue*

Tissue	Source	<i>Q</i> <sub>O<sub>2</sub></sub>	Reference
Lymph node	Rabbit	8.4	Unpublished, this laboratory
Lymph node	Human	5.7	(1)
Lymph node	Mouse	5.45	(2)
Lymph node	Mouse	6.3	(3)
Hyperplastic tonsil	Man	9.0	(4)
Thymus	Rat	6.0	(4)
Appendix	Rabbit	8.05	This paper
Lymphocytes	Rat	5	(6)
Lymphocytes	Man	6	(9)

tissues obtained previously (Table I). The  $Q_{O_2}$  values were similar to those obtained in this laboratory with rabbit lymph nodes, and those with tonsils reported by Warburg *et al.* (4).

### Glucose Metabolism

1.  $O_2$  Uptake. On addition of glucose to the Ringer-phosphate solution, there was a small increase in the  $O_2$  uptake (12%). The respiratory quotient in the presence of glucose approached 1 (0.93) (Table II).

2. Glycolysis. In the absence of  $O_2$  the anaerobic glycolysis (chemical determination of lactic acid) ( $Q_{L^{N_2}}$ , 10) was not as high as that of the mucous membrane of rabbit's large intestine reported by Rosenthal

TABLE II  
Effect of Glucose on the Respiration of Rabbit Appendix  
(Figures give cu. mm./mg./hr.)

Expt. no.	$Q_{O_2}$		$Q_{CO_2}$
	Control	Glucose	
I	7.04	8.84	
II	7.14	8.60	
III	7.50	7.81	
IV	7.35	8.20	
V	6.04	6.90	
VI	6.20	7.02	
		8.0	7.35
		7.63	7.02
		7.89	7.56

and Lasnitzki (22) ( $Q_{CO_2}^{N_2}$ , 12.6). Measurements of this anaerobic glycolysis by the manometric technique gave the same values, *i.e.*,  $CO_2$  formation in Ringer-bicarbonate:  $N_2:CO_2$  was entirely due to lactic acid. In the presence of oxygen, lactic acid formation was rather large ( $Q_{L^{O_2}}$ , 5.3) when compared with the aerobic glycolysis of the rabbit's large intestine ( $Q_{L^{O_2}}$ , 1.4) (Table III). This large aerobic glycolysis, indicative of impaired oxidative pathway of carbohydrate metabolism, seems to be characteristic of blood cells and of lymphatic tissue. However, the relatively high value of respiration combined with the relatively low value of anaerobic glycolysis puts this tissue among those



described by Warburg (23) as possessing a negative value of excess fermentation [excess fermentation =  $Q_L^{N_2} - (2 Q_{O_2})$ ]. The "excess fermentation" value in appendix,  $-5.4$ , compares well with the value of  $-4$  obtained for the thymus (rat).

3. *Pyruvate*. From the lactic acid found when the appendix was incubated in the presence of glucose and oxygen, it can be seen that even in optimum conditions for oxidation, the pyruvate formed during the obligatory anaerobic phase of glucose metabolism is partly reduced to lactic acid. When pyruvate was added to the Ringer-phosphate solution and the appendix was incubated in the presence of  $O_2$  as gas phase,

TABLE III

*Glycolysis in Rabbit Appendix*

$Q_L^{N_2}$ , and  $Q_L^{O_2}$  values give cu. mm. of lactic acid formed/mg. dry weight/hr.

Expt. no.	$Q_L^{O_2}$	$Q_L^{N_2}$
1	4.5	8.3
2	5.0	9.0
3	8.5	10.3
4	6.7	11.3
5	4.0	9.9
6	4.6	11.0
7	4.1	10.4
Average	$5.34 \pm 1.27$	$10.03 \pm 0.86$

there was utilization of 2.4 cu. mm./mg./hr. Although this is a small amount compared with pyruvate utilization by tissues of similar respiratory rate, it seems that *added* pyruvate is metabolized mainly by the oxidative pathway. In fact, the utilization of pyruvate in  $N_2$  as gas phase was very small,  $Q_{pyruvate}^{N_2}$ , 0.6, thus giving a high oxydismutation coefficient

$$\left( \frac{\text{Pyruvate utilized in } O_2}{\text{Pyruvate utilized in } N_2} = 5.8 \right) \text{ (Table IV).}$$

It has been maintained by some investigators that pyruvate can carboxylate and thus form oxalacetate (the so-called  $CO_2$  fixation) and that this oxalacetate might condense with pyruvate to form citrate. An increase in the  $CO_2$  tension would increase the rate of these reactions. To test this possibility the appendix was incubated in Ringer-phosphate solution with  $O_2$  as gas phase, and in Ringer-bicarbonate

TABLE IV  
*Pyruvate Metabolism of Rabbit Appendix*

Expt. no.	$Q_{O_2}$		$Q_{O_2}^{pyruvate}$	$Q_{N_2}^{pyruvate}$
	Control	Pyruvate		
I	7.52	7.67	1.01	
II	7.22	8.01	1.01	
III	8.70	9.70	3.00	0.4
IV	8.80	9.70	3.30	0.5
V			2.25	
VI			1.78	
VII			3.53	0.73
VIII			3.30	0.93

with  $O_2:CO_2$  as gas phase ( $5\% CO_2$  and  $20\% CO_2$ ). Increased  $CO_2$  tension had no effect on the utilization of pyruvate (Table V).

4. *Acetate.* The oxidative pathway of pyruvate ends in acetate formation. It was therefore necessary to study the metabolism of acetate. The  $O_2$  uptake was increased  $12\%$  in the presence of sodium acetate, and  $17\%$  in the presence of magnesium acetate. Accordingly, the utilization of sodium acetate ( $Q$  acetate, 1.54) was less than that of magnesium acetate ( $Q$  acetate, 2.5). The increase of acetate oxidation by  $Mg^{++}$  was shown by Lynen (24) in yeast, and by Kalnitsky and Barron (25) in rabbit kidney. Fluoroacetate inhibited both the  $O_2$  uptake and the acetate utilization (Table VI). In animal tissues it seems that the oxidation of acetate starts by its condensation with oxalacetate to form citric acid. Such a condensation was found to take

TABLE V  
*Effect of  $CO_2$  Tension on Pyruvate Utilization*  
 $Q$  pyruvate values, cu. mm. pyruvate mg./hr.

$O_2:CO_2$	$Q$ Pyruvate
100:0	2.25
100:0	1.78
100:0	3.17
100:0	3.08
95:5	2.04
95:5	2.13
80:20	2.50
80:20	2.46

place in pigeon liver by Stern and Ochoa (26) and in rabbit kidney by Rudolph and Barron (27). When appendix slices were incubated for half an hour with pyruvate and oxalacetate there was the formation of 0.13 cu. mm. of citric acid/mg. (2.3  $\mu$ g./hr.). When the tissue was incubated with acetate and oxalacetate, there was 0.15 cu. mm. of citric acid formation (2.6  $\mu$ g. hr.). Addition of adenosinetriphosphate (0.002 M) had no influence, probably because of phosphatase action and restrictions by membrane permeability.

TABLE VI  
*Acetate Metabolism in Rabbit Appendix*

Substrate	$Q_{O_2}$			$Q$ Acetate	$Q$ Acetate (FA)
	Control	Acetate	FA		
Sodium acetate	8.80	9.13		1.19	
Sodium acetate	8.65	9.40		1.09	
Sodium acetate	7.70	8.65		1.86	
Sodium acetate	7.50	9.12		1.79	
Magnesium acetate	6.93	9.45	2.60	1.5	0.4
Magnesium acetate	7.25	8.75	3.10	1.9	0
Magnesium acetate	7.20	8.12	2.68	3.0	0.3
Magnesium acetate	7.50	8.03	2.85	3.5	0.6
Magnesium acetate	8.34	9.30		2.0	
Magnesium acetate	8.55	9.80		2.2	

5. *Tri- and Dicarboxylic Acids.* The oxidation of citric acid requires its transformation into isocitric acid by aconitase. Aconitase activity in this tissue was measured in the homogenized tissue, according to the technique of Johnson (21) (Fig. 1). The  $Q$  aconitase value (cu. mm. citric acid formed/mg. dry weight/hr.) was 20. Johnson found a  $Q$  aconitase value of 61 for rat liver, 10 for brain, and 7.6 for testis.

Addition of citric and  $\alpha$ -ketoglutaric acids to appendix slices had little effect on the  $O_2$  uptake. Furthermore, the utilization of these substrates was small when compared with their utilization by other mammalian tissues (28). Addition of succinate increased the  $O_2$  uptake by only 17%. This increase was canceled on addition of malonate. Oxalacetate produced almost no effect, while the addition of magnesium acetate and oxalacetate increased the  $O_2$  uptake as much as magnesium

acetate alone. Undoubtedly in all these experiments the role of the membrane has to be considered as of primary importance. When the tissue was ground and succinic dehydrogenase activity was measured in the ground tissue by its oxidation with ferricyanide in Ringer-bicarbonate and  $N_2:CO_2$  as the gas phase (Fig. 2) there was steady oxidation, as shown by the  $CO_2$  formation. This oxidation was completely inhibited by malonate. The  $Q$  succinate value, 3.1, is low, although it compares well with the value of 1.6 obtained by Elliott and Greig in thymus (29).

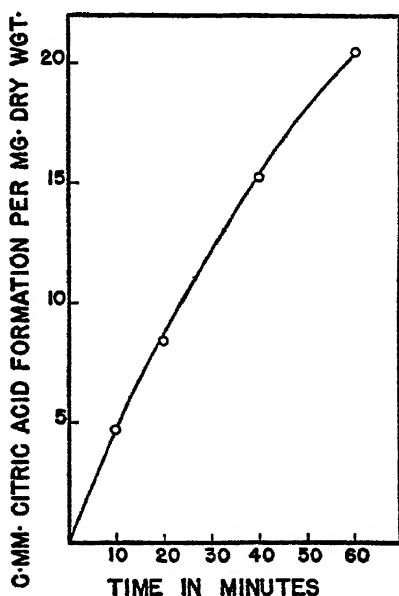


FIG. 1. Aconitase in rabbit appendix. Two g. of appendix was ground with Merek sea sand in 10 ml. phosphate buffer, pH 7.4, 0.1  $M$ , and centrifuged. Three ml. of the supernatant was added directly to 2 ml. of 0.0625  $M$  cisaconitic acid to give a final concentration of 0.025  $M$ . Incubated at 38°.

### *Amino Acid Metabolism*

As indices of amino acid oxidation, DL-alanine and L-glutamate were chosen because they are the amino acids oxidized at the highest speed by the D-amino acid and L-amino acid oxidases prepared from kidney or liver. Neither of them increased clearly the  $O_2$  uptake of appendix

slices. This slight action was confirmed by the measurement of  $\text{NH}_3$  formation. Glutamate gave a  $Q_{\text{NH}_3}$  of 0.13, and alanine, 0.15, values extremely low when compared with those given by the liver or the kidney. However, similar low values were observed by Goldinger *et al.* in the bone marrow (30). Probably in lymphatic tissue the oxidative pathway of amino acid metabolism is negligible and the amino acids

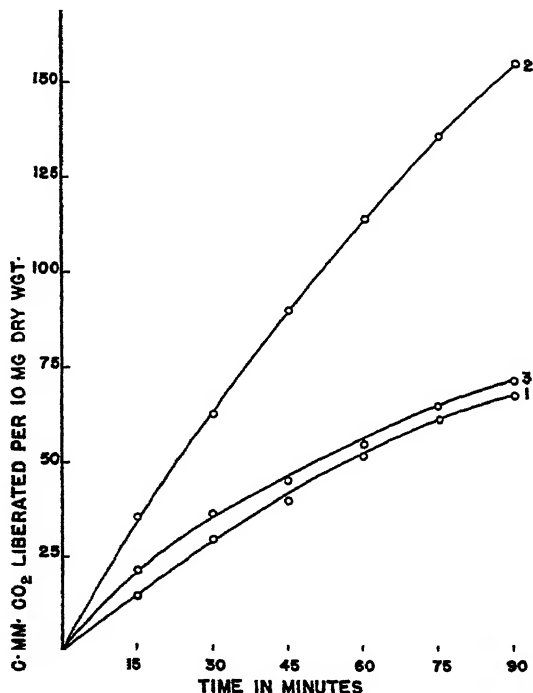


FIG. 2. Succinic dehydrogenase activity of appendix. 1. Control. 2. Succinate 0.01 *M*. 3. Succinate 0.01 *M* + malonate 0.03 *M*.

formed on amination of dicarboxylic acids or on hydrolysis of proteins undergo transaminations. This last reaction was studied in appendix slices, the transamination reaction measured being: glutamate + oxalacetate  $\rightleftharpoons$  aspartate +  $\alpha$ -ketoglutarate. In Ringer-phosphate buffer, of the 0.01 *M* glutamate added, 14% had disappeared in 15 min., and 48% in 60 min. (Fig. 3). The  $Q$  transaminase value, as calculated

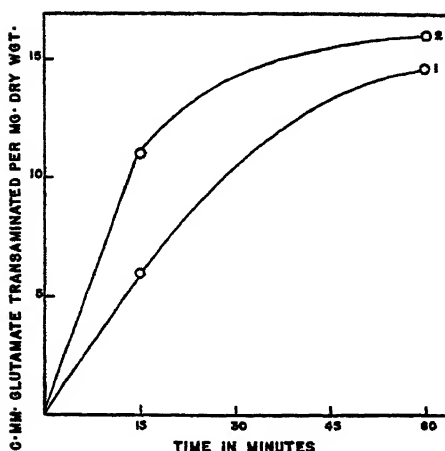


FIG. 3. Transamination by rabbit tissue slices. Incubated at 38°C. in Ringer-phosphate with oxalacetate 0.01 *M* and glutamate 0.01 *M*. Aliquots taken at 15 and 60 min. 1. Appendix slices. 2. Kidney cortex slices.

from the glutamic acid used in 15 min., was 24. This value is high for tissue slices, as kidney slices gave figures only slightly higher. It may be concluded that there is a vigorous transamination reaction in appendix slices which is possibly utilized for the synthesis of protein.

### *Fatty Acid Metabolism*

Addition of butyrate to appendix slices produced the same increase in the  $O_2$  uptake as succinate or acetate addition (17%). The utilization of butyrate (*Q* butyrate, 2.0) compared well with the values for butyrate utilization in guinea pig kidney (*Q* butyrate, 1.86) obtained by Kleinzeller (31). Jowett and Quastel (32) found that butyrate oxidation in liver slices was inhibited by malonate. Butyrate utilization by the appendix was largely inhibited by malonate, an indication that the

TABLE VII  
*The Metabolism of Butyrate in Rabbit Appendix*

Substrate	$Q_{O_2}$	<i>Q</i> Butyrate
None	7.96	
Butyrate	9.29	2.0
Butyrate + malonate	7.75	0.2
Butyrate + fluoroacetate	2.57	0

oxidation of butyrate must proceed via acetic acid and the tricarboxylic acid cycle. Malonate would not only stop completion of oxidation, but also by avoiding further formation of oxalacetic acid would inhibit the condensation process with acetic acid. Fluoroacetate inhibited butyrate utilization completely (Table VII). Further evidence that butyrate oxidation proceeds via formation of  $C_2$  fragments was given by the lack of acetoacetate formation on incubation of appendix slices with butyrate. No acetoacetate was detected on incubation of butyrate plus fluoroacetate or on incubation with caproate. Bartlett and Barron (33) found an increased accumulation of acetoacetate in the liver on incubation with butyrate and fluoroacetate. Addition of  $NH_4Cl$ , which increases the formation of ketone bodies in liver slices (34), had no effect at all.

TABLE VIII  
*Effect of Fatty Acids on the Respiration of Appendix*

Substance	Concentration	Increase in respiration		
		Per cent		
		First hr.	Second hr.	Third hr.
	<i>M</i>			
Caproate	0.01	+5	+20	+28
Caproate	0.001	+8	+15	+10
Caprate	0.01	-79	-94	-99
Caprate	0.001	+16	+23	+24
Stearate	0.001	-8	-7	-15
Stearate	0.0001	-5	-4	-8

Addition of caproate produced a steady increase in the  $O_2$  uptake of appendix slices, an indication of its oxidation. Caprate at the usual concentration of 0.01 *M* inhibited respiration almost completely, and at a concentration of 0.001 *M* increased it as much as caproate. Stearate, 0.001 *M*, inhibited the respiration slightly; with this fatty acid no increase in  $O_2$  uptake was found even when the concentration was decreased to 0.0001 *M* (Table VIII). The mechanism of this inhibition of the  $O_2$  uptake by relatively high concentrations (0.01 *M*) of high C-number fatty acids is still unknown. It cannot be attributed to their adsorption into the cellular membranes, for the same inhibition is observed in ground tissues. Perhaps they combine with the  $Mg^{++}$

and  $\text{Ca}^{++}$  ions, which are necessary for some of the oxidations, and form with them insoluble salts.

### SUMMARY

The metabolism of the appendix of rabbit has been studied to provide information on the metabolic activities of lymphatic tissue. The average  $Q_{O_2}$  value, 8.05, was similar to that of rabbit's lymph nodes. In the presence of glucose the respiratory quotient was 0.93. Aerobic glycolysis was found to be half as high as the anaerobic glycolysis, an indication of impaired pathway of carbohydrate oxidation. Pyruvate utilization was mainly via oxidation, as its metabolism in anaerobiosis was very small. Acetate was rapidly utilized by the tissue. Its metabolism is probably through citric acid formation by condensation with oxalacetate, for there was synthesis of citrate on addition of oxalacetate. The presence of active aconitase was established. The small utilization of citric and  $\alpha$ -ketoglutaric acids may be due to barriers interposed by the cell membranes. Succinic dehydrogenase activity was low. Slight, too, was the oxidation of amino acids, while transamination was active. Fatty acids were rapidly utilized by the appendix.

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# The Negative Effect of Iodinated Casein and Hog Thyroid to Effect a Conversion of Carotene to Vitamin A *in Vitro*<sup>1</sup>

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## INTRODUCTION

Many investigators have indicated that some relationship exists between the metabolism of vitamin A and the thyroid gland (1, 2). A direct relationship was suggested by Balaba (3) who reported that a brei of cattle thyroid gland or thyroglobulin had the carotenase activity that had originally been postulated for liver by Olcott and McCann (4). More recently, Kaplansky and Balaba (5) have reported that carotene is converted into vitamin A by incubation in the presence of iodinated casein or thyroglobulin. The present study is an attempt to confirm and extend the original observation of Balaba. After we began this work, Cama and Goodwin (6) reported that they were unable to confirm the effect of iodinated casein.

## EXPERIMENTAL

Iodinated casein, containing approximately 10% thyroxine, was dissolved in 0.0008 N NaOH and this was diluted to provide 50  $\mu$ g./flask. Each flask also contained 150  $\mu$ g. of carotene<sup>4</sup> and 2.5 mg. of tocopherol, dispersed with the aid of "Tween 80."<sup>5</sup> The pH was adjusted to 7.3, and the mixture was incubated anaerobically for 2 hr. at 37.5°C. The material was then saponified, the carotene and any vitamin A extracted, and the Carr-Price reaction applied. The carotene was determined by direct spectrophotometry, so that the Carr-Price values could be corrected for carotene. The details of the analytical procedure used have been reported by Mattson *et al.* (7). Controls

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<sup>4</sup> Valley Vitamins Company.

<sup>5</sup> Atlas Powder Company.

TABLE I  
Average Carotene Recoveries and "Apparent Vitamin A" Resulting  
from Incubating Various Solutions

Incubation flask	Number of experiments	Carotene	Length of incubation	Carotene recovered, mean	I.U. "apparent vitamin A." (See text Mean)
		$\mu\text{g.}$	$\text{hr.}$	$\mu\text{g.}$	$\mu\text{g.}$
0.0008 N NaOH	2	150	2	119	(2.5)
"Tween 80" + 50	2	0	2	(0.04)	(0.02)
I <sub>2</sub> Casein					
Casein 50	2	150	2	147	(4.9)
Boiled I <sub>2</sub> Casein 50	2	150	2	116	(3.4)
I <sub>2</sub> Casein 50	2	150	6	104	(13.4)
I <sub>2</sub> Casein 50	3	150	19	65.7	(1.7)
I <sub>2</sub> Casein 50	12	150	2	105.7	(7.8)
I <sub>2</sub> Casein 50	2	600	2	625	(9.4)

TABLE II  
"Apparent Vitamin A" Produced by Incubation of Thyroid  
Slices and Brei with Carotene

Incubation flask	Number of experiments	Carotene	Carotene recovered, mean	I.U. "Apparent vitamin A." (See text) Mean
		$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$
Thyroid homogenate	2	0	0	(1.0)
Thyroid homogenate	5	112	45.3	(4.9)
Thyroid slices	2	0	0	(2.1)
Thyroid slices	5	112	22.2	(5.1)
Ringer-Locke alone	3	112	46.8	(2.3)

consisted of uninodinated casein, boiled iodinated casein, and an equivalent amount of the 0.0008 N NaOH. The results are presented in Table I. The "apparent vitamin A" represents the difference between the Carr-Price value, and that calculated to be due to carotene.

A second group of experiments was carried out with homogenates and slices of thyroid gland. In this case, an attempt was made to make conditions more nearly physiological, and carotene suspensions were prepared in water. Carotene<sup>a</sup> was dissolved in methanol, water was added slowly during refluxing, and the methanol was finally distilled off. A solution containing 11.2  $\mu\text{g.}$  of carotene/ml. was obtained, and

<sup>a</sup> 10% alpha, 90% beta, General Biochemicals Company.

was stabilized by the addition of ascorbic acid. Fresh hog thyroid tissue in Ringer-Locke solution was employed, with additions of the carotene suspension in water. The pH was again 7.3, the temperature 37.5°C., and incubation was carried out under anaerobic conditions. The results are given in Table II.

### DISCUSSION

The incubation was carried out under anaerobic conditions because of the instability of carotene suspensions in the presence of oxygen. Even so, a considerable loss of carotene is noted in almost all of the experiments, suggesting that residual oxygen in the solutions was causing oxidation. There is reason to believe, then, that the carotene which remained after incubation might be somewhat altered. Since Johnson and Baumann (8) have shown that isomerization and partial oxidation of carotene can give rise to products which give a more intense Carr-Price reaction in relation to the absorption at 456 m $\mu$  than carotene does, it appears that "apparent vitamin A" recorded in the present experiments may be due entirely to changes in the carotene other than conversion to vitamin A. A change of approximately 10% in the ratio of Carr-Price intensity to "yellow color" would be sufficient to account for the "apparent vitamin A" values.

No fading of the blue color produced in the Carr-Price reaction was seen, though this is not of great significance, since it would have been expected that about 90% of the color (due to carotene) should be stable. Although the Russian workers report material with an absorption maximum at 328 m $\mu$ , we have been unable to detect any indication of such a maximum in Skelly-solve extracts of the saponified material from incubation with iodinated casein. When such an extract was chromatographed on a column of calcium hydroxide and Fuller's earth (1:1), two zones were observed which exhibit a somewhat greenish fluorescence. When these zones were eluted separately, no indication of a peak at 328 m $\mu$  was obtained in either.

Although it is difficult to prove that very small amounts of vitamin A have not been formed, we feel that our experiments must be interpreted as indicating that no significant amount of carotene is converted to vitamin A by iodinated casein or thyroid tissue under the conditions which we have employed.

### SUMMARY

We have been unable to confirm reports that iodinated casein, thyroid homogenate, or thyroid slices transform carotene into vitamin A.

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# Chemical Composition of Rabbit Bone Marrow in Benzene Poisoning

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## INTRODUCTION

Many morphologic studies of bone marrow in benzene intoxication have been reported, but few data are available on the chemical changes. The effect of benzene upon marrow depends upon the duration of exposure of the animal to the intoxicant, the intensity of the treatment, the age of the animal, and other factors (1). Hence, several effects such as aplasia, selective hypoplasia, or hyperplasia may be obtained (2). Li *et al.* (3) found that the fat content of the marrow of dogs receiving benzene was reduced to as low as 5%, but they gave no actual analyses, and did not correlate this with other chemical changes. This paper deals with the correlation of the gross constituents of bone marrow in rabbits in several degrees of injury produced by benzene injections, and includes analyses of the nitrogen, sulfur, and phosphorus components.

## EXPERIMENTAL

Rabbits, varying in age from 4 to 9 months, were given subcutaneous injections, twice daily, of 5 parts benzene in 1 part olive oil (4). The animals received 75 mg. benzene, 100 g. of body weight daily. The administration was continued until the desired change in the total leucocyte content of the blood was obtained. The injection of benzene was stopped in some of the rabbits to permit recovery.

The animals were killed by a blow and exsanguination. Pieces of marrow with varying degrees of activity were removed for chemical analyses from 10 to 16 locations. Determinations were made for water, lipide, residue (lipide-free solids), and nitrogen (5). The regression equations were calculated for the composition of marrow samples from each rabbit as described previously (5). The symbols *W*, *L*, and *R* represent the water, lipide, and residue as percentage of the whole marrow. For additional analyses, protein-free filtrates (fraction soluble in 10% trichloroacetic acid) of the total marrow from shafts of one humerus and one femur were prepared. They were analyzed for phosphate, sulfur distribution, and non-protein nitrogen as previously described (6).

## RESULTS

A summary of experimental conditions is given in Table I. The animals were divided into groups depending upon the degree of benzene intoxication as measured by the blood leucocyte count at the time of death. The analyses of the combined humerus and femur marrow of each animal are given in Table II, and the gross compositions of marrow samples from 10 to 16 sites in each rabbit are graphed in the figures.

TABLE I

*Blood and Marrow Changes in Rabbits Receiving 75 mg. Benzene per 100 g. Body Weight Daily*

Rab. no.	Age, in months	Weight change	No. of marrow samples analyzed	No. of days injected	No. of days on experiment	Leucocytes/cu. mm.		Histologic changes in marrow
						Low	At death	
1	4.5	per cent -15	16	28	28	400	400	Marked hypoplasia to complete aplasia
2	8.0	-11	15	23	24	500	500	
3	7.0	-10	15	9	9	700	700	
4	7.0	-18	16	13	23	700	1,500	Moderately cellular and immature
5	4.5	-7	15	20	20	2,000	2,000	
6	5.0	3	14	16	16	2,600	3,000	
7	5.0	6	16	29	29	2,700	7,300	Hyperplasia and lack of maturation
8	8.5	-5	15	23	41	1,700	5,300	Normal cellularity and an increase of mature cells
9	6.5	6	10	15	22	700	13,100	

The composition of the marrow was abnormal in all animals receiving benzene or showing a leucopenia at death. The major components showed a decrease in lipide and residue and an increase in water (Fig. 1). The decrease in residue was consistent with the decreased cellularity of the marrow, but the changes in water and lipide were not. Because of these changes, the composition of the marrow appeared to have shifted towards the active end of the graph in Fig. 1. This was due to the increased water content with the simultaneous loss of lipide (Fig. 2). The loss in residue was greatest in the animals with a leuco-

TABLE II

*Composition of Rabbit Marrow (Humerus and Femur) in Benzene Poisoning*  
Composition based on total marrow.

Rabbit no.	Water	Residue	Non-protein sulfur			Phos- phate	Nitrogen		
			Inorg. SO <sub>4</sub> <sup>-</sup>	Ether SO <sub>4</sub> <sup>-</sup>	Organic S		Total	Lipide	Non- protein
1	80.9	10.3	5.17	0.01	10.43	45.2	1.40	87	117.8
2	52.4	9.0	2.41	1.86	2.68	27.6	1.07	62	159.0
3	58.7	6.9	1.70	2.08	5.72	33.0	0.99	65	69.3
4	75.2	12.6	3.16	2.03	16.31	45.6	1.81	51	102.5
5	73.6	10.7	12.03	0	10.92	47.9	1.48	81	242.0
6	76.0	11.1	4.40	0.42	5.79	36.7	1.67	84	9.65
7	80.0	12.0	10.01	1.44	18.95	59.7	1.82	83	154.0
8	64.6	15.6	33.4	0	31.5	71.6	2.37	114	369.0
9	65.2	14.6	31.2	0	20.7	72.9	2.20	91	412.0
Normal av.	48.3	11.9	22.3	2.8	18.5	49.3	1.73	84	269.0

Cf. Table I for conditions. Normal average for 9 rabbits (6).

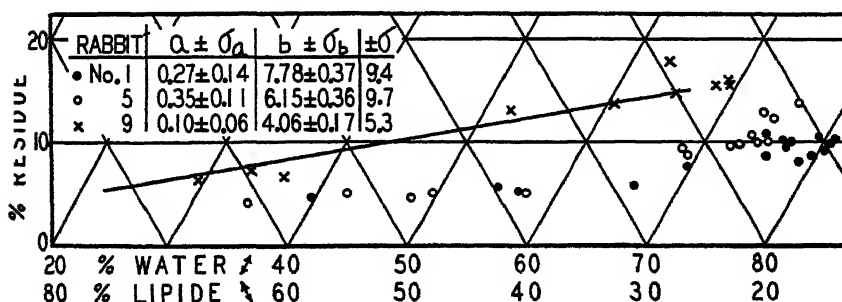


FIG. 1. Composition of bone marrow in benzene poisoning plotted on triangular coordinates. The length of the line shows the range of composition of marrow from various sites in normal rabbits of the same age (5). The values in the inset are the coefficients for the equation,  $W = aL + bR$ , and the standard errors of estimate. Cf. Table I, for experimental conditions.



penia of less than 1000 cells/cu. mm. blood, where, in the most active marrow, the residue content was reduced to 10% from a normal of about 15%. In the animals with leucocyte counts of 1000 to 3000, the loss was about one-half as much (Figs. 3 and 4). As in normal marrow (5), the water was associated with the residue. This is shown by the values of the coefficients in Fig. 1, where  $a$  and  $b$  represent the grams of water associated with each gram of lipid and residue, respectively. Thus in Rabbit 1, little or no water was associated with the lipid and  $7.78 \pm 0.37$  g. was associated with the residue. For normal marrow the value of  $b$  is  $4.24 \pm 0.07$ .

The changes in composition were not those of decreased activity as accompanies aging, which could have been expressed by the same re-

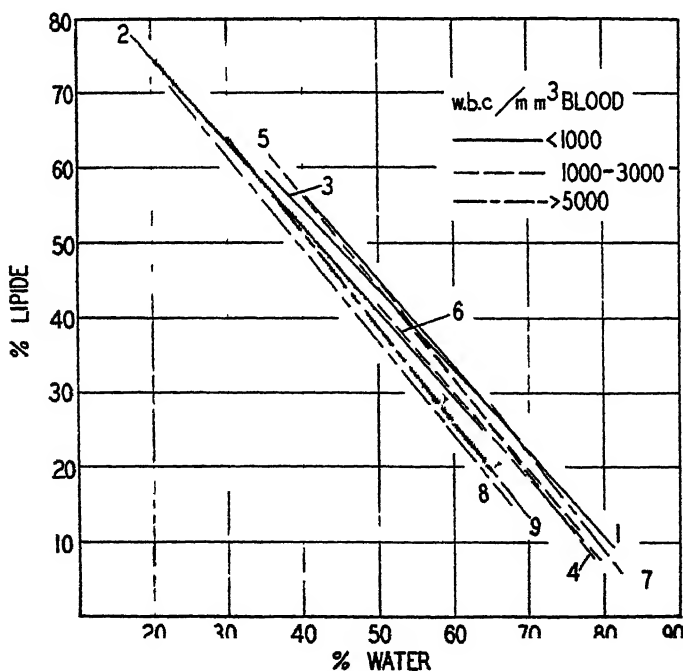


FIG. 2. Correlation of lipid and water in bone marrow in benzene poisoning. The lines are those calculated for the equation,  $L = a + bW$ , and their lengths represent the range of composition of 10 to 16 marrow samples in each animal. The shaded area shows the composition of normal rabbit marrow; its length represents the range of composition, and its sides one standard error of estimate (5). The standard errors of estimate of the other lines are of the same order of magnitude.

gression equation with a shift in limits. Many of the lines of composition paralleled the normal line, having about the same slope but different intercepts. In these cases, all marrow in the animal was affected proportionately. In Rabbits 2 and 3, Figs. 3 and 4, the slopes of the lines were changed indicating a somewhat greater action on the most active marrow. These two rabbits were older animals and the location of the lines of composition indicated the presence of a less active marrow, due to age, and a diminished effect of benzene as noted in older individuals (1).

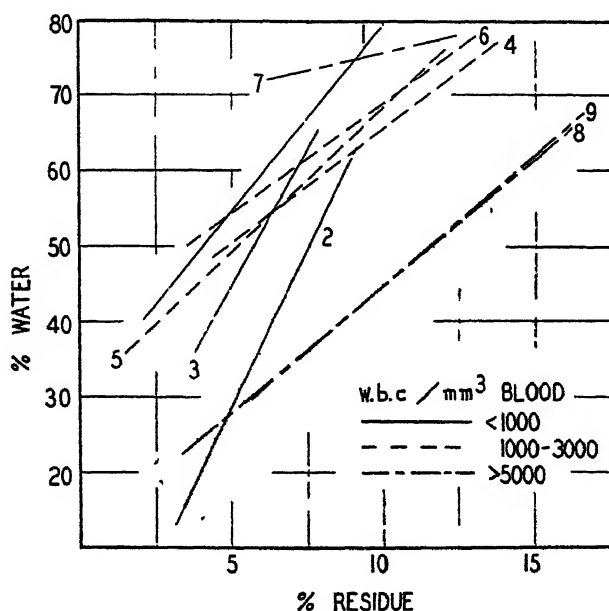


FIG. 3. Correlation of water and residue in bone marrow in benzene poisoning. The lines are those calculated for the equation,  $W = a + bR$ . Cf. Fig. 2.

The inorganic sulfate showed a very significant decrease in the leucopenic animals, Table II. There was, also, a marked loss in non-protein organic sulfur and non-protein nitrogen. The normal values for these components are variable (6), but the changes resulting from the benzene poisoning are so great there can be no doubt of their significance. The values for inorganic sulfate were in some cases lower than found in normal, inactive marrow of the radius and ulna (6). The inorganic

phosphate remained unchanged and as a result the ratio of phosphate to sulfate was increased. On an equivalent-weight basis, the ratio of inorganic phosphate-phosphorus to inorganic sulfate-sulfur was as high as 30 (normal, 2 to 7). With one exception this ratio was elevated in all the leucopenic animals. The distribution of non-protein sulfur showed an increase of the percentage ethereal sulfate in several cases but not in the absolute quantity.

In some animals, leucopenia could not be obtained. In other rabbits, there was a rapid recovery, even though benzene was being given

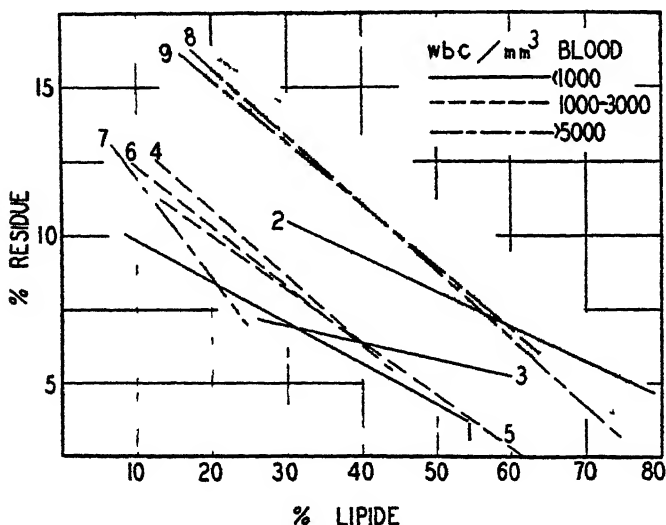


FIG. 4. Correlation of residue and lipid in bone marrow in benzene poisoning. The lines are those calculated for the equation,  $R = a + bL$ . Cf. Fig. 2.

Weiskotten and Steensland (7) have shown this to follow the development of an acute infection. Rabbit 7 illustrates this condition. The peripheral leucocyte count returned to normal while the benzene was being administered. However, the gross composition of the bone marrow showed the effects of benzene intoxication in the deviations from normal of the lines of composition (Figs. 2, 3, and 4). The shortness of the lines in the graphs representing the marrow of this rabbit, the position of the lines near the active ends of the graphs, and the histologic findings indicated that all marrow was highly active. Histologic examination showed the marrow of the radii and metatarsals to have con-

siderable activity. It was apparent that the full resources of the marrow were being utilized to overcome the adverse effects of benzene. The inorganic sulfate and non-protein nitrogen were low normal values. The other constituents were normal.

In Rabbits 8 and 9, the leucocyte counts were reduced by 80% and 90%, respectively. The benzene injections were stopped and the animals were allowed to recover. The marrow of these animals was somewhat more active than normal. The correlation equations were significantly the same as for normal marrow, but the limits were shifted showing that the marrow was higher than normal in water and residue and lower in lipide. The phosphate and sulfur and nitrogen components (Table II) were present in concentrations comparable to those found in a highly active normal marrow. This increased activity was apparently in response to the demand upon the animals for recovery. The ratios of phosphate to sulfate were normal.

The regression of total nitrogen on the residue content of the marrow remained normal in all animals. The calculated lines for these correlations, with one exception, fell within one standard deviation of the normal,  $R = 1.084 + 6.18 N$  (5). The lines of compositions, however, were shifted towards the inactive end as the percentages of total nitrogen and residue were lower than normal.

The regression lines of log of the lipide nitrogen (% of lipide) on the lipide were, in the majority of the leucopenic animals, between -1 and -2 standard deviations from the normal. The values were distributed at the active end of the normal line, indicating a loss of neutral fat, with a smaller or no loss in the "essential lipide" fraction.

Serum obtained at the time of death showed no significant changes in phosphate content or in non-protein sulfur content or distribution. The non-protein nitrogen was elevated to about 50 mg.-% in about half of the rabbits. This may have been due to a terminal congestion.

Urine analyses on some catheterized samples showed as much as 85% of the sulfur as conjugated sulfate. In most instances, this value was not above 25%. Outside of an increase in ethereal sulfate, no consistent changes in the daily excretion of sulfur, phosphate, or non-protein nitrogen could be demonstrated in urine samples collected in metabolism cages. During the benzene administration the volume of the urine was decreased, and it was voided at very irregular intervals.

## DISCUSSION

Radiation exposure and benzene intoxication are frequently compared for their action in causing hematopoietic changes (8, 9). In comparing the results obtained in this paper with those obtained for irradiated marrow (10), certain differences may be noted. The effects of benzene were more uniform on both active and inactive marrow than were those of X-radiation, where the most pronounced action was upon the active marrow. In both cases the lipide-free solid content was re-

duced. In the benzene intoxication, the water content of the marrow increased and the lipide showed a decrease, whereas the opposite was noted in the irradiated marrow. In both cases, a marked decrease in the sulfate and non-protein sulfur was found. In the benzene-treated animals, this may result from the function of sulfate in the detoxication of benzene, but it is questionable if this is the only factor as substantial amounts of inorganic sulfate were excreted in the urine. Radiation is known to act on the mercapto groups in enzyme systems (11). The reduction of the sulfur constituents of the marrow would indicate that both radiation and benzene alter the sulfur metabolism. That their action is somewhat different in mode or degree is indicated by the more rapid recovery in the cases of the benzene poisoning.

### SUMMARY

Rabbits were given 75 mg. benzene 100 g. body weight daily in two subcutaneous injections. The marrow was analyzed at several degrees of injury. The action of benzene on the marrow was to increase its water content and to decrease the amount of lipide, residue, total and non-protein nitrogen, and non-protein sulfur fractions. The ratio of nitrogen to residue was not altered, and the phosphate concentration remained normal. Animals which were permitted to recover from the benzene intoxication, until the marrow and blood leucocyte counts were normal, had marrow that was essentially normal in chemical composition. A comparison was made with the changes caused by X-rays.

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## Thermal Enzymes. II. Cytochromes<sup>1</sup>

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### INTRODUCTION

In a previous paper (1) we showed that a thermophilic bacterium (designated as No. 2184) possessed an enzyme, malic dehydrogenase, that resisted destruction by heat at 65°C. The malic enzyme seemed to be concentrated in an insoluble fraction which we termed the "red fraction."

In this paper we report on the cytochromes found in the red fraction. The cytochrome system of the red fraction, like the malic dehydrogenase, possesses a heat stability that is not shared by the cytochrome system from mesophilic bacteria or animal tissues.

### EXPERIMENTAL

#### *Preparation of the Red Fraction*

One-g. samples of thermophile No. 2184 (1) were weighed into 3 separate test tubes. To each tube was added 3 ml. of 1% lysozyme solution and 2 ml. of 0.9% sodium chloride. The cells were mixed immediately with the liquids to prevent clumping, and the viscous liquid was then stirred well with a Potter homogenizer. Four ml. more of the salt solution was added to each tube and the cell mixture again stirred with the homogenizer. Then another 4 ml. of the saline solution was added and the stirring repeated. The test tubes were then stoppered and placed in a water bath at 37°C. for 1-2 hr.

The combined lysate from 3 test tubes was centrifuged for 30 min. at 2500 r.p.m. to remove debris. The supernatant liquid was discarded as well as any dark debris on the

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very bottom of the centrifuge tube. The middle red layer was mixed with an equal volume of phosphate buffer of pH 7.4 and centrifuged for 12 min. at maximum on an International Centrifuge multispeed head. The supernatant and debris were discarded, and the red layer was again mixed with an equal volume of the phosphate buffer and again centrifuged for 12 min. at high speed. The supernatant liquid was discarded and the resultant red layer was used as the "red fraction." The yield was 1-1.5 ml. of concentrated red fraction.

### *Heat Treatment*

A sample of the red fraction (or other preparation) was pipetted into a 13 × 100-mm. pyrex test tube, which was then tightly stoppered and immersed in a constant temperature bath at 65°C. At definite intervals, aliquots were withdrawn and immediately cooled. The samples were then pipetted into the Warburg vessels and the activity for cytochrome oxidase was determined at 37°C.

### *Cytochrome Oxidase from Beef Heart*

This preparation was made according to the method of Stotz and others (2). The final product was free of cytochrome c when tested for oxygen consumption with hydroquinone.

### *Determination of Cytochrome Activity*

To each Warburg vessel was added the following: 1.5 ml. of *M*, 15 phosphate buffer of pH 7.2, 0.5 ml. of red fraction (or beef heart cytochrome oxidase), 0.5 ml. of *p*-phenylenediamine or hydroquinone (0.1 *M*), and enough water to make a total of 3.0 ml.

Cytochrome c, when added, was introduced as 0.4 ml. of a  $10^{-4}$  *M* solution. Extracts supposedly containing cytochrome c were added in 0.5-ml. quantities.

Controls without substrate and determinations of the autoxidation of hydroquinone always accompanied each run. Autoxidation of *p*-phenylenediamine was negligible.

### *Extraction of Cytochrome C*

Three ml. of concentrated red fraction was diluted to 25 ml. with water and a uniform suspension prepared. The suspension was then treated according to the method of Potter and DuBois (4) for the extraction of cytochrome c from tissues. The final extract was added to cytochrome c-free beef heart cytochrome oxidase, and the oxygen uptake determined using hydroquinone as substrate.

## RESULTS

We have shown before (1) that the malic dehydrogenase of thermophile No. 2184 is concentrated in the red fraction and that the enzyme possesses a heat stability that is not shared by the malic dehydrogenase

TABLE I

*Cytochrome Oxidase Activity of Red Fraction from Thermophile No. 2184*

Preparation	$\mu\text{l./30 min./mg. N}$
Red fraction	1
Red fraction plus <i>p</i> -phenylenediamine	134
Red fraction plus hydroquinone	133
Red fraction plus hydroquinone plus cytochrome c	114

of mesophilic bacteria and kidney tissue. The red fraction of thermophile No. 2184 consists of an insoluble layer of protein matter separating from the solutions of the organisms after lysis with lysozyme. At high speeds, the fraction separates in a pink uniform layer that can be removed from the centrifuge tube with a spatula.

The red fraction also contains cytochromes. This was shown by the uptake of oxygen when *p*-phenylenediamine and hydroquinone were used as substrates (Table I).

Furthermore, the cytochromes of the red fraction of the thermophile were stable to heat to a degree that is not the case with the cytochrome system of mesophilic bacteria and of heart tissue. In Fig. 1 the heat stabilities of these three sources of the enzymes are compared. At 65°C. the animal cytochrome system and the mesophilic bacterial system were destroyed in a few minutes, whereas the thermophile enzyme was destroyed only slowly in 90 min. to an extent of 50%.

The question then became one of establishing the identity, or the lack of identity, of the cytochromes of the red fraction with well known cytochromes of animal tissues.

Cytochrome b seemed to be absent from the red fraction. This was first shown by the comparison of the oxygen uptakes with *p*-phenylenediamine and hydroquinone. Cytochrome b does not oxidize hydroquinone (2) but does oxidize the diamine. Hence the difference between the two oxidations can be a rough measure for the presence of cytochrome b. The red fraction showed little difference in the oxygen con-

TABLE II

*Cytochrome Oxidase Activity of Purified Beef Heart Oxidase*

Preparation	$\mu\text{l./30 min./mg. N}$
Beef heart oxidase	3
Beef heart oxidase plus <i>p</i> -phenylenediamine	289
Beef heart oxidase plus <i>p</i> -phenylenediamine plus cytochrome c	600
Beef heart oxidase plus hydroquinone	14
Beef heart oxidase plus hydroquinone plus cytochrome c	188



sumption for these two substrates over a series of different runs, whereas a preparation of cytochrome oxidase from beef heart gave a significant difference which was presumably due to the cytochrome b that accompanied the oxidase (Table II).

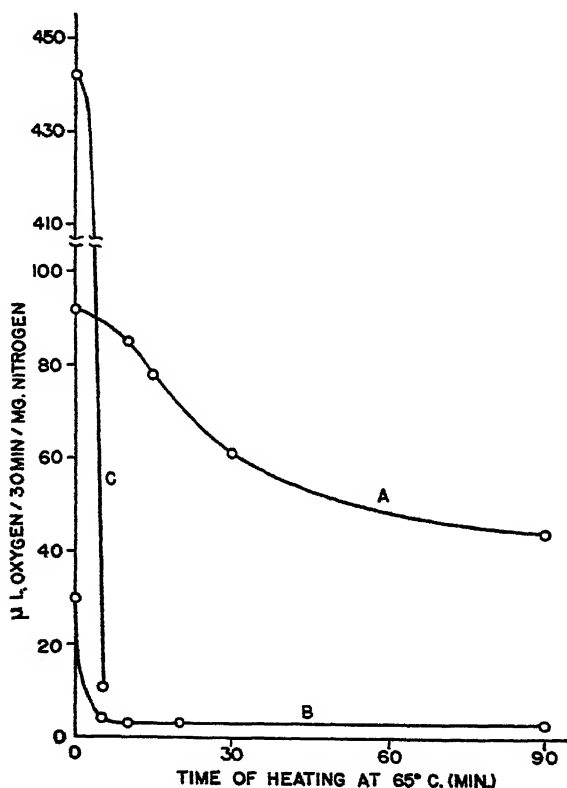


FIG. 1. Activity of cytochrome system after heating at 65°C.; A, red fraction from thermophile No. 2184; B, cell lysate from a mesophile; C, cytochrome oxidase from beef heart. The substrate was *p*-phenylenediamine.

Further, the cytochrome system of the red fraction was inhibited by levels of cyanide that normally inhibit only cytochrome oxidase but do not inhibit cytochrome b. At  $5 \times 10^{-6} M$  the inhibition was about 60%, and at  $1 \times 10^{-4} M$  the inhibition was complete for the oxidation of hydroquinone (see Fig. 2). With *p*-phenylenediamine as substrate, the inhibition was complete at a concentration of cyanide

equal to  $5 \times 10^{-4} M$ . Stotz and others (2), also Keilin (3), found that cytochrome b was not affected by concentrations of this order. They state that only very high concentrations of cyanide will inhibit cytochrome b.

Cyanide inhibition also demonstrates that some form of cytochrome oxidase must be present in the red fraction, since the cytochromes do not react with low concentrations of cyanide. Cytochrome oxidase seems to be the limiting enzyme of the cytochrome chain, since an addition of cytochrome c did not increase the activity (Table I). If cytochrome c limited the red fraction, the addition should have

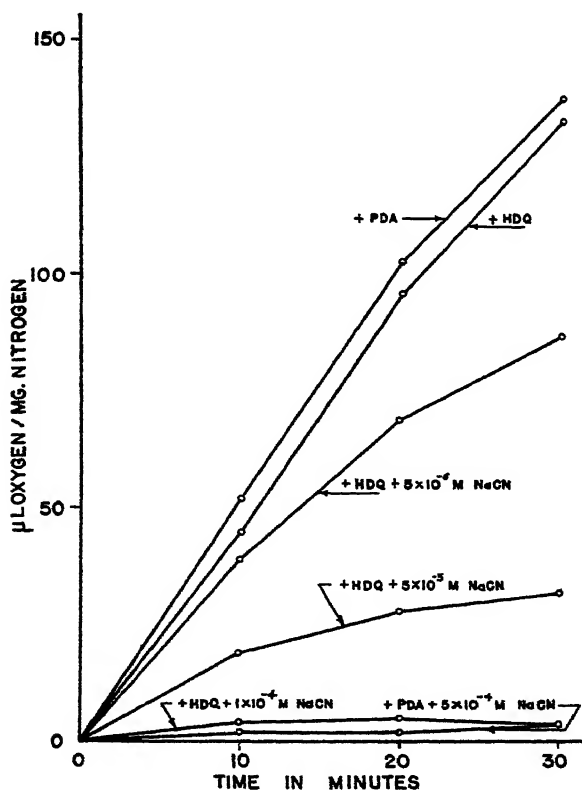


FIG. 2. Inhibition by cyanide of cytochrome oxidase of the red fraction. To each Warburg vessel was added 1.4 ml. of  $M/15$  phosphate buffer of pH 7.2, enough sodium cyanide to bring the flask contents to the desired molarity, 0.5 ml. of red fraction, 0.5 ml. of 0.1  $M$  *p*-phenylenediamine or hydroquinone, and water to bring the total volume up to 3.0 ml.

enhanced activity, provided that the added cytochrome *c* penetrated the particles of the red fraction.

The spectroscope easily confirmed the enzymatic data for the presence of cytochrome *c*. Even without the addition of sodium hydrosulfite, the bands of reduced cytochrome *c* could be detected in a suspension of the red fraction. On reduction with sodium hydrosulfite, the bands became clearer and coincided completely with the bands of a commercial preparation of reduced cytochrome *c* from beef heart. No bands other than the cytochrome *c* bands could be detected.

TABLE III

*Cytochrome Oxidase Activity of the Red Fraction and of Different Preparations from the Red Fraction*

Preparation	Substrate	$\mu$ l. 30 min./mg. N
Red fraction <sup>a</sup>	<i>p</i> -Phenylenediamine	90
Red fraction ppt. at pH 4.5	<i>p</i> -Phenylenediamine	40
Red fraction ppt. at pH 4.5 plus cyto. <i>c</i>	<i>p</i> -Phenylenediamine	40
Boiled solution of red fraction plus beef heart oxidase <sup>b</sup>	<i>p</i> -Phenylenediamine	14
Trichloroacetic acid extract of red fraction plus beef heart oxidase	Hydroquinone	2

<sup>a</sup> The red fraction lost some activity by precipitation. The original activity was not restored by adding cytochrome *c*.

<sup>b</sup> The beef heart oxidase prepared for this experiment had a low oxygen uptake with *p*-phenylenediamine alone. With added cytochrome *c* it gave excellent consumptions. Usually, beef heart preparations give high uptakes with *p*-phenylenediamine, so that one is forced to use hydroquinone as a substrate when assaying for cytochrome *c*.

The cytochrome *c* of the red fraction was resistant to solution, whereas the cytochrome *c* of beef heart dissolves readily. A precipitation at pH 4.5 with acetate buffer, similar to the procedure of Stotz, Sidwell, and Hogness (2) for the freeing of beef heart oxidase of cytochrome *c*, did not remove cytochrome *c* from the red fraction (Table III). Boiling did not release the cytochrome from the red fraction; and the conventional method for the extraction of cytochrome *c* from tissues with trichloroacetic acid (4) failed to remove it. Table III shows the results from various attempts to release cytochrome *c* from the red fraction using purified cytochrome oxidase from beef heart as the test enzyme for the presence of cytochrome *c* in solution.

Further evidence that the cytochrome *c* of the red fraction was not available for solution was shown by combining red fraction and puri-

fied beef oxidase. If the cytochrome *c* of the red fraction had been available for use by the beef oxidase, the oxygen uptake should have been greater than the sum of the independent consumptions. This was not the case. Figure 3 shows that the oxygen uptake was no greater than that of red fraction without the beef oxidase. We conclude from this that the cytochrome *c* of the red fraction is tightly bound to the protein of the red fraction.

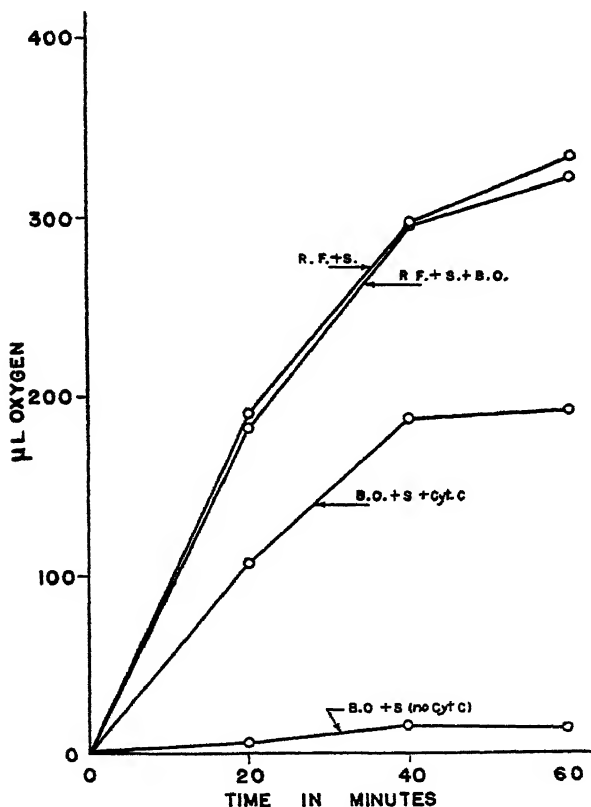


FIG. 3. Cytochrome oxidase activity of various preparations of red fraction and of beef heart; substrate (*S*) hydroquinone; red fraction (*R.F.*) from thermophile No. 2184; beef heart oxidase (*B.O.*).

All values were corrected for the autoxidation of hydroquinone. Hydroquinone was used as the substrate, since the beef heart oxidase could not be purified sufficiently to bring its oxidation of *p*-phenylenediamine (without added cytochrome *c*) down to small values.

## DISCUSSION

Whether, in thermophile No. 2184, we are dealing with exactly the same cytochromes isolated from animal tissues is difficult to say. The cytochrome oxidase of the thermophile has one important difference from the usual oxidase, that of heat stability. It resembles the other, however, in its inhibition by cyanide, insolubility in buffer solutions, and in its response to the usual substrates of the cytochrome system. Cytochrome c of the thermophile has exactly the same spectral lines of animal cytochrome c but differs in being difficult to bring into solution. We are unable to say whether this insolubility is due to a high molecular weight or to a binding on the part of the proteins of the red fraction, since thus far we have not been able to prepare the cytochrome c from the thermophile.

As yet, a general statement regarding the heat stability of thermophilic enzymes should not be made from our data. To be sure, the malic dehydrogenase and the cytochrome system of the red fraction are quite stable at the temperature of the growing organism (65°C.). This stability does not mean that the inherent structure of the protein molecule possesses the stability. The red fraction behaves like a compact mass of protein which in some way resembles an organization of molecules. Perhaps the red fraction represents a unit of protoplasm that is released intact from the bacterium on lysis with lysozyme. If such is the case, we could logically expect a heat stability for it that may not at all be the case for the purified enzymes.

## SUMMARY

The cytochrome system found in thermophile No. 2184 possesses heat stability at 65°C., the optimum growing temperature of the organism.

Cytochrome oxidase and cytochrome c appear in the red fraction isolated from the bacterium. The cytochrome c in the red fraction is bound tightly and cannot be released by heating or by extraction with trichloroacetic acid.

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# The Differential Heat, Alkali, and Trypsin Inactivation of Pancreatic Lipase

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## INTRODUCTION

It has been observed that pretreatment at 60°C. of pancreatic extracts causes a considerably greater reduction of the rate of the hydrolysis of methyl butyrate than of glycerides such as monobutyrin and olive oil (1). The purpose of the present study is to extend this observation to a greater variety of substrates and to investigate the possibilities of effecting a differential inactivation of the ester-hydrolyzing enzymes of pancreas.

The procedures have included the following: (a) differential heat-inactivation of glycerolated homogenates of pancreas, (b) differential alkali-inactivation of fractions of pancreas homogenates, and (c) the activity of the enzyme fractions toward specific substrates after treatment with crystalline trypsin.

## EXPERIMENTAL

### *Substrates*

For the study of the pancreatic extracts and the fractions derived therefrom, the following were employed: Methyl butyrate, ethyl butyrate, propyl *n*-butyrate, *n*-butyl *n*-butyrate, *n*-butyl propionate, ethyl oleate, monobutyrin, glycol dipropionate, tributyrin, tripropionin, and olive oil. Ethyl oleate and monobutyrin were prepared according to methods described previously (2). The other esters were Eastman Kodak Co. products and were redistilled several times. Olive oil was purified according to the current U. S. Pharmacopoeia. The boiling points of the esters agreed with the values given in the literature.

The esters of the monovalent alcohols were used in a concentration of 2 mmoles/20 ml. of buffer solution, to which 6 ml. of the enzyme was subsequently added. The

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concentration of ethyl butyrate was adjusted to 1.14 mmole in the same volume. The esters of the trivalent glycerol were adjusted to a final concentration of 0.66 mmole: 20 ml., and the olive oil to a final concentration of 0.56 ml. 20 ml.<sup>1</sup> Glycol dipropionate was used in a concentration of 1 mmole 20 ml. No emulsifying agent was used for the latter as well as for methyl butyrate, ethyl butyrate and monobutyrin since they dissolve completely under these conditions. All the other esters, as well as olive oil, were emulsified with half their weight of gum arabic.

Two-tenths molar phosphate buffer of pH 8 was employed throughout the investigation. A range of optimal activity between pH 7 and 8 for both liver esterase and pancreatic lipase has been established by Sohotka and Glick (3).

### *Method of Estimation*

The formol titrimetric method was employed by which both the amount of acid liberated from the esters as well as the autolysis of the enzyme proteins was estimated. All the values obtained were therefore corrected by the enzyme and the substrate blanks. Titrations of 4-ml. aliquots were carried out with 0.05 *N* NaOH from a microburet (4 drops ca. 0.05 ml.). The results were reproducible within an error limit of about  $\pm 0.05$  ml.

### *Heat Inactivation of the Glycerol Extracts*

Hog pancreas freshly obtained from the slaughter house and stored at  $-20^{\circ}\text{C}$ . was homogenized in a Waring Blendor with 9 parts of a 90% glycerol solution. The glycerol homogenate was incubated for 16 hr. at  $30^{\circ}\text{C}$ . and then stored in a refrigerator at  $4^{\circ}\text{C}$ . For the heat-inactivation experiments, measured amounts of these extracts were transferred into 25-ml. graduates of 1-cm. diameter fitted with glass stoppers, and were brought up to the 7-ml. mark. The graduate was then transferred to a water bath at  $60^{\circ}\text{C}$ . and allowed to stand for 25 min. The maximal temperature fluctuation did not exceed  $0.5^{\circ}$ . Thereafter the graduate was placed for 5 min. into ice water. The volume of the glycerol extract was again checked and twice its amount of buffer was added. The graduate was then stoppered and the contents thoroughly mixed. Aliquots were removed and brought with buffer to the final dilution. The unheated control was treated likewise. The results of inactivation by pretreatment at  $60^{\circ}\text{C}$ . reveal the markedly greater reduction in the initial rates of the hydrolysis of the monovalent alcohol esters and of glycol dipropionate compared with that of the glycerides (Table I). Figure 1 shows the effect on the hydrolysis of methyl butyrate and of tributyrin by pretreatment of glycerolated pancreas homogenates at different temperatures.<sup>2</sup>

### *Alkali Inactivation of Hog Pancreas Preparations*

Hog pancreas was defrosted, defatted, and treated in a Waring Blendor with two volumes of ice-cold distilled water. The homogenate was then centrifuged at 3000

<sup>1</sup> Since the exact molecular weight of olive oil is unknown, its concentration was adjusted so as to equal the saponification value given by 0.33 mmole of glycerol triesters.

<sup>2</sup> In this, as in the following experiments, concentration of the enzymes was adjusted as to be proportional to their activity.

r.p.m. for 20 min. in a refrigerated centrifuge. The pellet was discarded and the supernatant liquid (Sc) was brought to an alcohol concentration of 7.5% and stored at  $-3^{\circ}$  for 14 hr. The precipitate ( $P_1$ ) which was collected after centrifugation for 20 min. at  $26,000 \times g$  was suspended in a minimum of distilled water and dialyzed against distilled water for 16 hr. at  $5^{\circ}\text{C}$ . If not homogeneous in appearance, it was again dispersed in a small Waring Blendor. Reprecipitation of  $P_1$  produced a 25% decrease in enzymatic activity with respect to all the esters employed.<sup>2</sup>

TABLE I

*Influence of Heating on the Hydrolysis of the Esters by Glycerolated  
Pancreas Homogenate*

The glycerol homogenates were heated to  $60^{\circ}\text{C}$ . for 25 min. Values are based on the titration with 0.05 *N* NaOH of 4-ml. aliquots out of a total of 26 ml. enzyme-substrate mixture. Enzyme concentration 1.093 mg. *N*/ml. for the hydrolysis of all the glycerides, 0.312 mg. *N* ml. for the hydrolysis of methyl butyrate, and 0.364 mg. *N* ml. for the hydrolysis of the remaining esters.

Substrate	Activity in: <sup>a</sup>		Decrease in hydrolysis
	Unheated homogenate	Heated homogenate	
			<i>per cent</i>
Methyl butyrate	1016	68	93.4
Ethyl butyrate	171	0	100
Propyl <i>n</i> -butyrate	1092	68	93.8
<i>n</i> -Butyl <i>n</i> -butyrate	1092	0	100
<i>n</i> -Butyl acetate	172	0	100
Glycol dipropionate	418	60	85.6
Monobutyrim	304	100	67.2
Tributyrim	1094	444	59.3
Tripropionin	591	314	46.9
Olive oil	197	60	69.6

<sup>a</sup> Activities were estimated from the linear part of the curves and are expressed in micromoles of NaOH consumed/hr. mg. of enzyme nitrogen.

To 3-ml. aliquots of  $P_1$ , 0.05 ml. or 0.1 ml. of 0.5, 1.0, or 2 *N* NaOH was added. The pH of the solutions was immediately checked with a glass electrode, and the mixtures allowed to stand at room temperature for 30 min. Two-ml. aliquots of each were then added to 6 ml. of 0.2 *M* phosphate buffer at pH 7.6 and diluted with two to three

<sup>2</sup> The average activity obtained for  $P_1$  was 0.6 mmoles/mg. nitrogen of the enzyme/hr. incubation at  $38^{\circ}\text{C}$ . using methyl butyrate as substrate. Fractionation by alcohol precipitation was primarily aimed at purifying the homogenate or low molecular activators, which might not be removed by dialysis alone. It should be noted that differential centrifugation of liver or of pancreas homogenates at  $26,000 \times g$  failed to bring about a clear-cut increase in activity either in the pellets or in the supernates.



parts of buffer at pH 8.0, yielding a final pH of 8.0. A control tube containing  $P_1$  and water rather than NaOH was treated in a similar manner. After a preincubation of 6 min. at 38°C., these solutions were mixed with the substrates which were dissolved or emulsified as described above. The results are given in Fig. 2.<sup>4</sup>

### *Isolation of a Differentially Inactivated Protein Fraction from Pancreas by Trypsin Treatment*

The fraction  $P_1$  from pancreatic homogenate was prepared as described above and the protein-nitrogen concentration was adjusted to 5 mg. ml., and the pH adjusted to

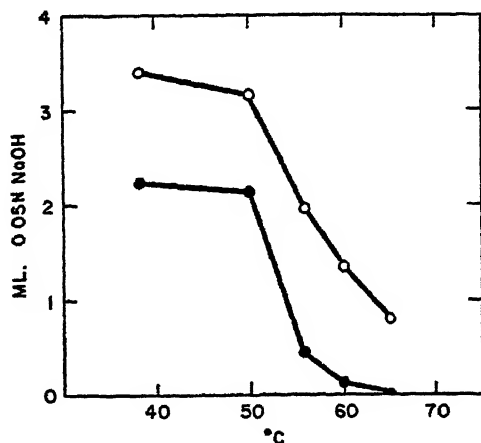


FIG. 1. Heat inactivation of pancreas glycerol homogenate at various temperatures. Concentration of substrates 0.33 mmole of tributyrin and 1 mmole of methyl butyrate in 10 ml. of phosphate buffer pH 8.0. Concentration of enzyme: 0.364 mg. of nitrogen ml. Values are based on the titration of 5-ml. aliquots out of a total of 13 ml. of enzyme-substrate mixture. Incubation: 20 min. at 38°C. ● Methylbutyrate. ○ Tributyrin.

7.4 with 5%  $\text{Na}_2\text{CO}_3$ . To 260 ml. of this was added 130 ml. of 0.2 *M* phosphate buffer of pH 8, yielding a final pH of 7.75. To this, 1.59 mg. of crystalline trypsin was added and the mixture was allowed to remain in an incubator at 38°C. for 3 hr.<sup>5</sup> Aliquots were taken initially and at various intervals in the course of the incubation, and were tested against methyl butyrate. The ratio of the rate of the hydrolysis of tributyrin

<sup>4</sup> Most preparations of  $P_1$  yielded results which were either identical or very close to those given in Fig. 1. In some instances, however, a markedly decreased alkali stability of  $P_1$  was observed, causing a shift of the slope of the inactivation curves to a lower pH interval (4). The cause of this different behavior of those fractions obtained under identical preparative conditions could not be ascertained.

<sup>5</sup> This trypsin preparation (Armour) contains approximately 50% magnesium sulfate.

to that of methyl butyrate was observed to change from an initial value of 2.1 to 3.4 after 150 min. of incubation. When the enzymatic hydrolysis of methyl butyrate was practically zero, the incubation was discontinued. The digest was then dialyzed at 5°C. against repeatedly renewed distilled water for 16 hr. Three hundred ml. of the dialyzed digest was then frozen and lyophilized. Four g. of a fluffy, slightly yellowish substance was obtained which readily dissolved in water (Fraction  $P_L$ ). Nitrogen determinations of a number of samples gave an average of 8.8%. The low nitrogen value suggests that a substantial amount of water was present. This preparation was

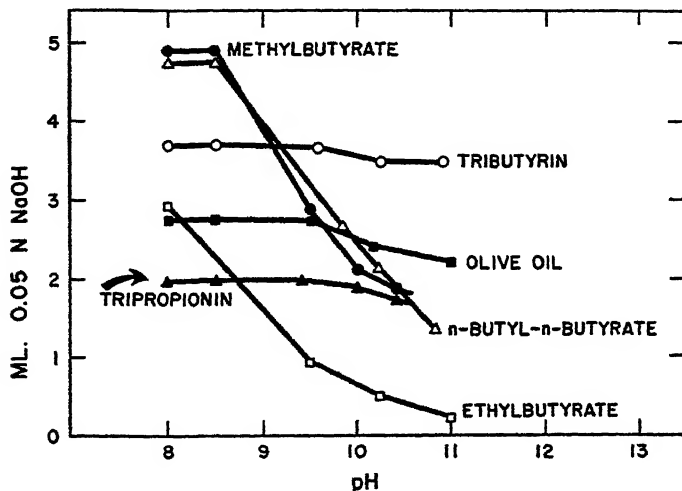


Fig. 2. Inactivation of fraction  $P_1$  by alkalinization. Concentration of substrates: 0.667 mmole of tributyrin and tripropionin, 0.58 ml. of olive oil and 2 mmoles of methyl butyrate, *n*-butyl *n*-butyrate and ethyl butyrate all in 20 ml. of phosphate buffer, pH 8.0. To each substrate was added 6 ml. of dilute  $P_1$ . Concentration of  $P_1$ : For all esters 0.56 mg. of N/ml., for olive oil 0.840 mg. N/ml. Incubation period: 60 min., incubation temperature: 38°C. Values were obtained by titration of 4-ml. aliquots. Essentially similar results were obtained with *n*-butyl propionate, propyl *n*-butyrate, ethyl oleate and glycol dipropionate, the inactivation curves of which all are similar to those of methyl butyrate, ethyl butyrate, and *n*-butyl *n*-butyrate.

dissolved in phosphate buffer at pH 8 and studied with various substrates. At a concentration of 0.511 mg. of nitrogen /ml. of enzyme solution, hydrolysis of tributyrin amounted to 30 and 45% after 20 and 40 min., respectively, whereas that of methyl butyrate was practically zero. It was possible to bring about a 9% hydrolysis of methyl butyrate in the course of a 20-min. incubation by trebling the enzyme nitrogen concentration. At this concentration, however, the initial velocity of the tributyrin hydrolysis was so high as to render its estimation practically impossible. The pretreatment of fraction  $P_1$  by trypsin under the conditions stated, therefore, does not com-

pletely destroy all capacity of the fraction to hydrolyze the monovalent ester, but does reduce it to a nearly negligible level in comparison with the capacity to hydrolyze the glycerides. Estimation of the relative rates of hydrolysis of the esters was therefore carried out at a nitrogen concentration of about 0.5 mg./ml.; in the original fraction  $P_1$  this easily allowed the estimation of the enzymatic activity towards both monovalent alcohol and glycerol esters, and in fraction  $P_L$  that toward the

TABLE II

*Initial Rates of Hydrolysis and Hydrolysis after 40 Min.  
of the Esters by Fractions  $P_1$  and  $P_L$*

Concentration of the triglycerides 0.66 mmole/20 ml. buffer. Concentration of glycol dipropionate 1 mmole and of the monoesters 2 mmoles in 20 ml. of buffer. Enzyme nitrogen concentration: Fraction  $P_L$ : For tributyrin and methyl butyrate 0.511 mg./ml., for tripropionin and *n*-butyl *n*-butyrate 0.525, for triheptylin and methyl laurate 0.538, for glycol dipropionate 0.500, for monobutylin and triacetin 0.407, and for olive oil 0.671 mg. of nitrogen/ml. Fraction  $P_1$ : For olive oil 0.608 mg. ml. and for all the other esters 0.507 mg./ml. Four-ml. aliquots of the enzyme-substrate mixture were removed for titration.

Substrates	Initial rates of hydrolysis by fraction: <sup>a</sup>		Decrease in hydrolysis by fraction $P_L$ <sup>b</sup>
	$P_1$	$P_L$	
			<i>per cent</i>
Monobutylin	1064	156	85.4
Tributylin	2950	920	68.9
Triacetin	272	0	100
Tripropionin	2223	2217	0
Triheptylin	994	400	57.8
Olive oil	310	122	60.7
Methyl butyrate	700	30	95.7
<i>n</i> -Butyl <i>n</i> -butyrate	840	60	92.9
Methyl laurate	210	0	100
Glycol dipropionate	460	62	86.6

<sup>a</sup> Initial rates of activity were calculated from the linear part of the curves and are expressed as micromoles of NaOH consumed hr. mg. of enzyme nitrogen.

<sup>b</sup> Hydrolysis by fraction  $P_L$  of the glycerides lacks the high *initial* rate of velocity characteristic for their hydrolysis as shown by fraction  $P_1$ . In terms, however, of per cent hydrolysis after 40 min. of incubation, when the cleavage of the glycerides by fraction  $P_1$  reaches its maximum, the per cent losses in hydrolysis by fraction  $P_L$  amount to 25 for monobutylin, 30 for tributyrin, 34 for triheptylin and 49 for olive oil, and are thus considerably smaller than the activity losses calculated from the initial rates of hydrolysis.

glycerides. Activities were computed from the linear part of the time curves. The results are given in Table II.<sup>6</sup>

### *Studies on Liver*

These studies have been extended in similar fashion to hog liver homogenates. Such homogenates as well as fractions prepared from them were considerably less inactivated by heat or alkali treatment and not inactivated at all by treatment with crystalline trypsin as compared with their pancreas analogs. The decrease in the rates of the hydrolysis of any of the esters employed caused by either heating of the glycerolated extract to 60°C. or by alkali pretreatment to a pH of 10 never exceeded 25% and no substrate specific inactivation effects were noticed. In view of the failure of the liver preparations to be appreciably inactivated under conditions similar to those employed with the pancreas analogs, a complete description of the experiments is omitted for reasons of brevity.

### DISCUSSION OF THE RESULTS

Inactivation experiments by heat and by alkali treatment clearly demonstrate that with pancreas a sharp line can be drawn between the behavior of the esters of monovalent alcohols and of the bivalent glycol on the one hand, and that of the trivalent glycerol esters on the other. The hydrolysis of the former, following the heat treatment of the homogenate, is reduced nearly to zero, whereas that of the triglycerides is considerably less affected. Pretreatment with heat of the homogenate reduces primarily the initial rates of hydrolysis of the glycerides, whereas in terms of per cent hydrolysis achieved after 40 min. of incubation the degree of inactivation is considerably less in most instances (Table I).

Alkali treatment of fraction  $P_1$  causes a decline of hydrolysis of the monovalent esters and of glycol dipropionate which is dependent upon the pH at which the enzyme was pretreated, whereas the hydrolysis of the triglycerides remains almost unchanged (Fig. 2).

It is evident from the data in Table II that pretreatment with crystalline trypsin of fraction  $P_1$  reduces markedly its enzymatic activity toward methyl butyrate. The total hydrolysis of methyl butyrate with  $P_L$  amounts to only 6.6% as compared with 52% in  $P_1$ .

\* Triacetin, triheptylin, and methyl laurate have not been employed previously and have been chosen in this particular instance for the sake of a still greater variety of substrates. The former two were used in a concentration of 0.33 mmole, 20 ml. of buffer solution. The concentration of methyl laurate was adjusted to 2 mmoles in the same volume. No gum arabic was used for triacetin which dissolves easily under those conditions.

In fraction  $P_L$ , enzymatic activity toward all the monovalent alcohol esters and toward glycol dipropionate has been greatly reduced.

It is, however, of interest to note that triacetin, in contrast with all the other glycerides studied, is not hydrolyzed by fraction  $P_L$ . Enzymatic specificity in this instance is apparently partly determined by the acid residue, for triacetin is hydrolyzed by both fraction  $P_1$  and the crude pancreas homogenate. These sources fail however to hydrolyze two- and three-carbon-chain esters of ethanol such as ethyl acetate and ethyl propionate.

The evidence obtained thus strongly suggests the coexistence in pancreatic extracts of at least two enzymes concerned with the hydrolysis of the monovalent alcohol esters, the glycol esters, and triacetin on the one hand, and of the homologous glycerol ester series beginning with tripropionin on the other.

#### ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. J. P. Greenstein for the interest, help, and encouragement extended by him throughout this investigation.

#### SUMMARY

The heat, alkali, and trypsin inactivation of hog pancreas homogenates and of fractions thereof has been investigated.

A fractionation of the pancreatic extract could be made whereby one fraction which was relatively thermostable and which acted upon the glycerides and another which acted upon the monovalent alcohol and glycol esters were discerned.

Treatment of fractions of pancreas homogenates at various degrees of alkalinity produced evidence for a separation of the activities in the pancreas preparations toward the glycerides on the one hand, and toward the monovalent alcohol and glycol esters on the other.

By incubation of pancreas fractions with crystalline trypsin the hydrolysis of the monovalent alcohol esters and triacetin was reduced nearly to zero, whereas the decrease of the extent of the hydrolysis of all the other triglycerides amounted to no more than 69%. The decrease in the hydrolysis of glycol dipropionate as affected either by heat treatment or by incubation with trypsin was larger than that of the glycerides but smaller than that suffered by the monovalent alcohol esters.

A protein fraction in pancreas homogenates which had been differentially inactivated by treatment with crystalline trypsin could be isolated.

The coexistence in pancreatic extracts of at least two different ester-hydrolyzing enzymes is suggested.

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# Isolation of Xanthine, Guanine, Adenine, Proteose, Oxalic Acid, and Glutathione from Peanut Kernels

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The peanut kernel was examined for the presence of some minor constituents to gain further information on its qualitative composition. The compounds isolated and identified were found in the supernatant liquid from the preparation of protein from peanut kernels. The composition of this liquid has a bearing on the purity of the isolated protein and is of interest in connection with its possible use as a nutrient for yeast (1) and other microorganisms.

## EXPERIMENTAL

### *Isolation of Xanthine, Guanine, and Adenine*

About 21 kg. of hexane-extracted meal from peanut kernels yielded 120 l. of supernatant liquid after precipitation of the protein with sulfur dioxide as described previously (2). After addition of lead acetate to remove most of the sulfur dioxide, the supernatant liquid was fractionally precipitated with mercuric sulfate. The second fraction, after removal of the mercury, was evaporated *in vacuo* and fractionated further by the addition of ethanol. One of the fractions gave a strong Weidel's test for xanthine, and after purification with dilute ammonium hydroxide weighed 147 mg. Its x-ray diffraction pattern agreed with that of a known sample of xanthine.

*Analysis*— $C_5H_4O_2N_4$ . Calculated: C, 39.5; H, 2.7; N, 36.8. Found: C, 39.1; H, 3.0; N, 36.9.

Another fraction, purified by precipitation with cuprous oxide, weighed 1.2 g. and proved to be crude guanine. It was purified by solution in water, precipitation with a few drops of ammonium hydroxide solution, and conversion to the hydrochloride. After conversion back to free guanine the yield was 85 mg.

*Analysis*— $C_5H_5ON_5$ . Calculated: C, 39.7; H, 3.3; N, 46.3. Found: C, 39.2; H, 3.5; N, 44.6.

The x-ray diffraction pattern of the gold chloride double salt agreed with that of the gold chloride double salt of guanine.

<sup>1</sup> One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

*Analysis*— $C_5H_5ON_5 \cdot HCl \cdot AuCl_3$ . Calculated: N, 14.3; Au, 40.1. Found: N, 14.1; Au, 39.3.

By addition of an excess of hydrochloric acid to the concentrated supernatant liquid from the crude guanine, a crystalline product was obtained which yielded 11 mg. of the gold chloride double salt of adenine. This was identified by microscopic comparison of its crystallographic properties with a known preparation and by x-ray diffraction pattern.

### *Isolation of a Proteose*

Peanut meal, 13 kg., was extracted in a manner similar to that used above except that sulfuric acid rather than sulfur dioxide was used to precipitate the protein. An excess of saturated lead acetate was added to the supernatant liquid from the protein precipitation. The precipitate was used for the isolation of oxalic acid described below. The supernatant liquid was precipitated with an excess of mercuric sulfate in 20% sulfuric acid and the mercury removed from the precipitate with hydrogen sulfide. After evaporation under reduced pressure the filtrate was poured into a large excess of ethanol. The precipitate weighed 40 g. It was purified by dissolving in water, removing the remaining sulfuric acid with aminized cotton fabric (3), and precipitating again with alcohol.

The purified proteose did not precipitate from water solution by a change in pH. It could be precipitated with trichloroacetic acid but not with nitric acid. A water solution formed a precipitate with picric acid which dissolved on warming and appeared again on cooling. The proteose dialyzed through a collodion membrane. It contained 18.6% nitrogen, 2.1% cystine, 0.56% methionine, and 0.03% phosphorus on a dry basis. Two-dimensional paper-partition chromatography (4) on a hydrolyzed sample indicated the presence of 16 amino acids.

### *Isolation of Oxalic Acid*

The lead precipitate from the proteose isolation was de-leaded with hydrogen sulfide, concentrated, and precipitated with an excess of ethanol. The alcoholic supernatant liquid was evaporated to a sirup which was extracted with ethyl ether. On evaporation of the ether 125 mg. of oxalic acid was obtained. The acetamide oxalate (5) melted at 127.5° (cor.), mixed melting point 127.5°. After purification of the oxalic acid and recrystallization from water it was analyzed.

*Analysis*— $C_2H_2O_4 \cdot 2H_2O$ . Calculated: C, 19.1; H, 4.8. Found: C, 19.1; H, 4.8.

Peanut meal was found to contain 0.16% oxalic acid by the method of Pucher, Vickery, and Wakeman (6).

### *Isolation of Glutathione*

Shelled peanut kernels, 9 kg., were extracted with 70% ethanol in a Waring Blendor. After concentration of the alcoholic extract under reduced pressure, 43 mg. of glutathione was isolated by the method of Hopkins (7). Paper-partition chromatograms of the material before and after hydrolysis corresponded with those from a known sample of glutathione. The sulfur content was 10.8%. Calculated for glutathione, 10.4%.



The glutathione content of the peanut kernels by a method specific for non-protein mercapto compounds (8) was  $0.024 \pm 0\%$ . Non-protein mercapto compounds other than glutathione may be present in peanut kernels.

#### ACKNOWLEDGMENTS

We are indebted to Elizabeth R. McCall for the oxalic acid determination, to Merrill E. Jefferson and Mildred D. Murray for the microscopic and x-ray identifications, and to Lawrence E. Brown for the microanalyses.

#### SUMMARY

Xanthine, guanine, adenine, a proteose, and oxalic acid were isolated from the supernatant liquid remaining after the precipitation of peanut protein. Glutathione was isolated from an alcoholic extract of peanut kernels. These materials have not previously been reported to be constituents of peanut kernels.

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## LETTERS TO THE EDITORS

### Retardation of Tumor Growth in Mice by Oral Administration of Ethylenimine Derivatives<sup>1</sup>

Certain ethylenimine (aziridine) derivatives have been studied for their effect on the growth of tumors in mice. Triethylenimino-s-triazine and hexamethylenediethylenurea brought about considerable retardation of growth of transplanted tumors in mice when added to their diet in amounts of 0.001–0.15% by dry weight. The mice ate about 3 g. of the food a day. The per diem consumption for each was then from 0.03 mg. to 4.5 mg.

Mice of five strictly inbred strains were used. The influence of diets containing 0.001% of triethylenimino-s-triazine and hexamethylenediethylenurea was tested on the growth of five types of sarcomata and four types of carcinomata. The carcinomata included epithelioma of skin and bladder, mammary gland, and prostatic carcinomata. The retarding action of both drugs was more pronounced on the growth of carcinoma than sarcoma.

Oral administration of the several ethylenimines studied failed to prevent growth or to cause regression of native tumors transplanted in inbred mice of the same strain. Two of the compounds did prolong the life of tumor-bearing animals when administered in amounts on which the treated mice thrived and bore offspring.

Preliminary results with mice of the C57 strain implanted with sarcoma 241 according to the method described previously (1,2) and fed diets containing triethylenimino-s-triazine and hexamethylenediethylenurea are given in Table I.

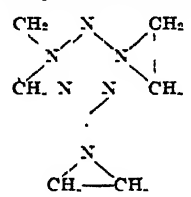
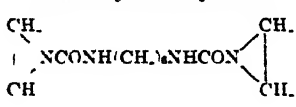
From the data in the table it will be seen that the compounds listed are decidedly effective in retarding the tumor growth. Triethylenimino-s-triazine appears to be the more effective of the two compounds.

Other compounds of the series showing retardation of tumor growth in the preliminary study are: 2,4-diamino-6-ethylenimino-s-triazine, 2-amino-4,6-diethylenimino-s-triazine, 2,4-diethylenimino-6-phenyl-s-

<sup>1</sup> Aided by a grant from the National Cancer Institute, Bethesda, Md.

TABLE I

*Retardation of Tumor Growth in Mice Using C57 Mice and Sarcoma No. 241 Implants*

Name and formula	No. of animals	Per cent in diet	Av. no. of days	Av. tumor size	
Triethylenimino-s-triazine 	9 7 5 10	0.001 0.005 0.0075 0.01	18 18 20 14	cu. mm. 1201 910 786 354	Toxic level—Here 6 animals died before 20 days.
Hexamethylenediethylenurea 	5 5 2 4 4 3	0.001 0.001 0.0015 0.005 0.010 0.025	17 30 15 13 15 7	1769 5621 2854 217 198 —	
Controls	24	—	15	16000-8000	

triazine, tolylene-2,4-diethylenurea, octadecylethylenurea, 2-ethylenimino-4,6-dimethoxy-s-triazine. The results obtained with the entire series will be published later.

An interesting, and possibly significant, observation was made from the post-mortem examinations on the animals used in some of these tests. No lung aberrations appeared in any of the mice used in these experiments, while mice of the same strain used in other experiments in the laboratory at the same time showed approximately twenty per cent incidence of lung carcinoma.

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Contribution from the laboratories of the  
 Wistar Institute of Anatomy and Biology,  
 Philadelphia, and from the laboratories of the  
 Calco Chemical Division of the American  
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## The Action of Certain Ethylenimine (Aziridine) Derivatives on Mouse Leukemia<sup>1</sup>

Because of their chemical reactivity certain ethylenimine derivatives and related compounds have been of interest to the textile industry (1). Their reactivity with cellulose, as well as their structural relationship to transformation products from the hydrolysis of nitrogen mustards, suggested an evaluation of their chemotherapeutic activity in experimental cancer. This preliminary report presents the results obtained in mouse leukemia. Studies on the pharmacology (2), activity against various solid tumors in mice and rats (3,4), against mouse tumor explanted to the chorio allantoic membrane of the chick embryo (5), and clinical evaluation (6) will be published later.

The technic for evaluation of the chemotherapeutic activity of a given drug by means of its ability to prolong the survival time of mice with transmitted leukemia has been described previously (7,8).

In a typical experiment, mice of the inbred Akm stock were injected intraperitoneally with 0.1 ml. of a saline suspension of leukemic spleen so diluted that 0.1 ml. contained 1,000,000 cells. Leukemia Ak4 (9), a relatively acute strain, was used. Forty-eight hours later, the mice were divided into comparable groups of ten mice each. Compounds were then given intraperitoneally in maximum tolerated doses three times weekly for ten doses.

The results of the regular screening experiments with 2,4,6-triethylenimino-*s*-triazine (SK 1133) are presented in Table I.

The presence of a small amount of water-insoluble polymer in the original sample led to its use initially suspended in 5% gum arabic in saline. Further studies by the Pharmacology Section (2) revealed that the insoluble fraction of the compound was only 2% by weight and that the remainder of the preparation could be used in saline. Although the gum arabic suspensions were active, the saline solutions were considerably more toxic and were effective at a fraction of the dose level of the gum arabic preparations. Further studies are in progress on SK 1133 and such related compounds as 2-amino-4,6-diethylenimino-*s*-triazine, hexamethylenediethylenurea, and tolylene-2,4-diethylenurea.

<sup>1</sup> This investigation was supported, in part, by a research grant from The National Cancer Institute of The National Institute of Health, United States Public Health Service, and, in part, by a research grant from The American Cancer Society.

SK 1133 has been shown to inhibit Sarcoma 180 (4) and several other tumors in mice and rats (3). The chemotherapeutic effects of this group of compounds appears to be due to the ethylenimine rings.

Reports have been received of several other laboratories independently studying these and other related compounds (10,11).

TABLE I  
*Experiments Using SK 1133*

Dose mg/kg.	Weight change		Survival time (Days)						Increase
			Untreated			Treated			
	Untreated <sup>a</sup>	Treated <sup>b</sup>	Number of mice	Range	Mean	Number of mice	Range	Mean	

SK 1133 suspended in 5% gum arabic in saline. One suspension used throughout entire course of injections.

	g.	g.							per cent
4	+2.3	-3.2	19	11-16	13.3	6	23-31	26.8	101
4	+0.8	+2.4	20	10-15	11.7	9	16-50	25.2	115
4	-1.8	-0.9	19	8-11	9.3	20	14-17	15.4	66
4	+1.6	-1.9	20	8-15	9.6	10	12-19	15.5	62
4	+4.2	-0.2	20	8-11	9.6	10	14-17	14.7	53

SK 1133 suspended in saline previous to each injection.

0.75	+4.7	-0.4 <sup>a</sup>	18	8-14	10.2	10	14-25	17.0	67
0.60	+1.1	-2.9 <sup>a</sup>	9	8-15	9.6	9	11-37	16.8	75
0.55	+2.1	+0.6 <sup>a</sup>	18	8-14	9.8	8	12-20	13.0	33

<sup>a</sup> Weight change calculated as the difference between the initial weight and that 1 week later.

<sup>b</sup> Weight change calculated as the difference between the initial weight and that 2 weeks later.

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## The Structure of Hydroxylysine

In connection with work on the metabolism of lysine we have worked out an isolation procedure for hydroxylysine and determined its structure.

Through the work of Van Slyke and his collaborators (1, 2) it appears probable that hydroxylysine is either  $\text{CH}_2(\text{NH}_2)\text{CH}(\text{OH})\cdot\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\cdot\text{COOH}$  (A) or  $\text{CH}_2(\text{OH})\text{CH}(\text{NH}_2)\cdot\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$  (B).

We have hydrolyzed a preparation of dried fishskin and isolated the fraction containing only lysine and hydroxylysine with the aid of ion exchangers. These amino acids were then separated by partition chromatography with 0.1 *N* hydrochloric acid supported on Hyflo Super-cel as the stationary phase and with phenol as the moving phase.

The fractions containing only hydroxylysine, as shown by filter paper chromatography, were combined and freed from phenol by extraction with ether. Fifty per cent of the nitrogen was liberated as ammonia with alkaline periodate (3). Benzoylation in strongly alkaline solution yielded a *N*-dibenzoate, m. p. 166-7°.

*Anal.* Calcd. (as  $\text{C}_{20}\text{H}_{22}\text{O}_6\text{N}_2$ , mol. wt. 370.4): C, 64.85; H, 5.99; N, 7.56. Found: C, 64.61, 65.00; H, 5.98, 5.88; N, 7.37, 7.35. Equiv. wt. 376.9.

Hydrolysis of the benzoate yielded a crystalline hydroxylysine sulfate.

When the dibenzoate was oxidized with chromic trioxide in 97.5% acetic acid at 50°, two oxidation equivalents were consumed corresponding to the oxidation of a secondary alcohol to a ketone. 1-Benz-

amido-2-octanol behaved in the same way whereas 2-benzamido-1-ethanol consumed four equivalents under the same conditions with the formation of hippuric acid. The structure of hydroxylysine thus corresponds to formula (A), *i.e.*,  $\alpha$ ,  $\epsilon$ -diamino- $\delta$ -hydroxyhexanoic acid.

To confirm this, we have reduced a small sample of hydroxylysine with hydriodic acid and red phosphorous at 160°. With filter-paper chromatography it was found that all hydroxylysine had disappeared whereas lysine had been formed in the reaction.

A full report will appear in due course.

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### Growth-Promoting Effect of Aureomycin on Pigs

Pigs on a diet consisting principally of yellow corn and peanut meal were found to grow slowly and to show no growth response to a supplement containing vitamin B<sub>12</sub> while in contrast an "animal protein factor" (APF) supplement produced a marked increase in growth (1). It has now been found that aureomycin will promote growth under similar conditions.

In Expt. 1, at Pearl River, the basal diet contained ground yellow corn 57%, peanut meal 41.5%, minerals and vitamins as described by Cunha *et al.* (1), with additional amounts of ferric citrate, manganous sulfate, potassium iodide, cupric sulfate, and cobalt acetate (2). Expt. 2 was carried out at the Florida Agricultural Experiment Station at Gainesville and the same diet was used as in Expt. 1 except that the trace minerals were modified slightly. Five Chester White pigs were used in each group in Expt. 1 and four Duroc pigs in each group in Expt. 2. The results are shown in Table I. A feeding period of 28 days was used. The APF supplement was prepared by drying the mycelium from deep aerobic cultures of *Streptomyces aureofaciens* together with

some filter-aid. The vitamin B<sub>12</sub> concentrate contained approximately equal parts of vitamins B<sub>12</sub> and B<sub>12b</sub> and had at least 10 mg. of vitamin B<sub>12</sub> activity per gram of solids.

TABLE I

Expt. no.	Group no.	Av. starting weight	Supplement kg. basal diet	Av. daily gain
		lb.		lb.
1	1	29.3	50 µg. vitamin B <sub>12</sub> (crystalline)	0.62*
	2	29.3	100 mg. aureomycin HCl (recrystallized)	0.87
	3	29.3	50 µg. vitamin B <sub>12</sub> plus 100 mg. aureomycin HCl	0.86
	4	29.2	20 g. APF supplement	0.88
2	1	35.6	44 µg. vitamin B <sub>12</sub> (as concentrate)	0.84*
	2	35.7	44 µg. vitamin B <sub>12</sub> plus 50 mg. aureomycin HCl	1.22
	3	35.6	44 µg. vitamin B <sub>12</sub> plus 200 mg. aureomycin HCl	1.51
	4	35.6	10 g. APF supplement	1.30

\* Diarrhea was observed in these groups.

Results with chicks (3) provided the first indication that a growth-promoting factor in addition to vitamin B<sub>12</sub> was produced by *Streptomyces aureofaciens*. The present experiments, as well as further results with chicks (4), indicate that aureomycin will produce a growth response when added to diets containing vitamins B<sub>12</sub> and B<sub>12b</sub>.

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## The Effect of Vitamin B<sub>12</sub>, Animal Protein Factor and Streptomycin on the Growth of Young Pigs <sup>1</sup>

Smith and Robinson (1) have shown that streptomycin markedly alters the intestinal flora of mice. Moore *et al.* (2) in a study of anti-bacterial agents in chicks, found that the inclusion of streptomycin in the diet, resulted in a significant growth response.

The basal ration used in the present experiment was composed of corn, 75%; solvent processed soybean oil meal, 22%; bonemeal, 1%; salt, 1%; and limestone, 1%. Vitamins A and D were added to the ration in amounts which supplied 2000 I. U. of vitamin A and 200 I. U. of vitamin D lb. of feed. In addition, the ration was supplemented with B vitamins in the following amounts (all values expressed in mg./lb. of feed): nicotinic acid, 15; calcium pantothenate, 10; and riboflavin, 2. The protein content of the basal ration was 18.1% by analysis.

TABLE I  
*Growth Response of Pigs Fed the Various Experimental Rations*  
(Six animals in each lot on trials lasting 6 weeks)

Lot no.	Supplement	Av. starting weight	Av. daily gain	Feed gain
		lb	lb.	lb. lb.
1	None	26	0.88	2.99
2	B <sub>12</sub> @ 12.5 µg., lb. feed <sup>a</sup>	26	0.93	3.04
3	B <sub>12</sub> @ 12.5 µg. lb. feed plus streptomycin @ 0.05% <sup>a</sup>	24	1.48	3.08
4	APF @ 0.5% <sup>b</sup>	24	1.43	3.07

<sup>a</sup> The crystalline vitamin B<sub>12</sub> and streptomycin (calcium chloride complex) was supplied by Merck & Co., Inc., Rahway, New Jersey.

<sup>b</sup> The dried APF material was supplied by Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

The growth response of the four experimental lots is shown in Table I. The addition of vitamin B<sub>12</sub> to the basal ration resulted in approximately a 10% increase in body weight gain as compared with the animals fed the basal ration. However, the addition of vitamin B<sub>12</sub> and streptomycin to the basal ration resulted in a 40% increase in average

<sup>1</sup> Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 1132.

daily gains. The addition of dried animal protein factor (APF) supplement also resulted in an increased average daily gain which was of approximately the same magnitude as the daily gain of the animals in lot 3. There was no difference of any significance between the four lots with respect to the efficiency with which the animals converted feed to body weight gain. It was further noted that feces of pigs in lots 1 and 2 were soft and pasty, a condition usually noted with corn-soybean type rations. However, the feces of pigs in lots 3 and 4 appeared to be of normal consistency.

If streptomycin alters the intestinal flora of the pig as it does in the case of the mouse and chick (1,2), it seems possible that this alteration is, in some way related to the increase in growth.

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### Vitamin B<sub>12</sub> and Methionine Formation

Recent studies have shown that an interrelationship exists between vitamin B<sub>12</sub> and transmethylation reactions. This vitamin has been demonstrated to protect weanling rats against kidney hemorrhage produced by a diet low in choline and methionine (1) and to reduce the choline requirement of young chicks (2). A vitamin B<sub>12</sub> concentrate has been shown to exert a lipotropic effect in rats maintained on a high-fat diet (3). The present communication reports on the differences observed in the methionine-forming system between the livers of vitamin B<sub>12</sub>-deficient and treated rats.

All of the animals were maintained on a diet containing soybean meal and 0.25% thyroid powder (Diet 286), previously employed by Emerson (4) in the production of vitamin B<sub>12</sub> deficiency. After an initial depletion period of 28 days, some of the rats were treated orally with 0.25 µg. of crystalline vitamin B<sub>12</sub> Merck daily. The animals were killed by decapitation; the livers were removed and homogenized with

TABLE I  
*Formation of Methionine by Rat Liver Homogenates*

Expt no	Days without B <sub>12</sub>	Days with B <sub>12</sub>	Methionine formed <sup>a</sup>	
			Choline + homocystine	Betaine -- homocystine
1	47	—	μg 220	μg 444
	28	19	345	480
2	56	—	243	
	28	28	381	
3	56	—	271	486
	38	28	394	740
4	60	—	261	
	28	32	336	
5	62	—	282	
	60	2	255	
	28	34	336	
6	64	—	290	340
	28	36	425	540
7	67	—	168	294
	60	7	303	411

<sup>a</sup> Methionine, μg./g wet weight liver. Each horizontal line represents data on a single rat, figures for methionine are averages of duplicate determinations.

2 parts of alkaline isotonic potassium chloride solution in an all-glass homogenizer. The procedures described by Borsook and Dubnoff (5, and Dubnoff (6) for study of methionine formation were modified as follows: Aliquots (1.5 ml. or 2.0 ml.) of the homogenates were added to 0.1 ml. 0.1M phosphate buffer pH 7, contained in 20 ml. rubber-stoppered bottles. In addition, one or more of the following solutions was present: 0.4 ml. 0.1 M choline, 0.4 ml. 0.1 M betaine, and 0.2 ml. 0.05 M homocystine. Water was added to make a final volume of 4.7 ml. The mixtures were gassed vigorously with nitrogen for 1 min.; the stoppers were replaced and the bottles incubated with shaking for 3 hr. in a 37° water bath. After incubation, 0.3 ml. of 100% trichloroacetic acid was

added, and the precipitate removed by filtration. A 2.0 ml. sample of the filtrate was analyzed for methionine by Borsook and Dubnoff's modification (5) of the McCarthy-Sullivan reaction (7). The amount of methionine present was determined from a standard curve (against a zero time control) employing a Beckman spectrophotometer, Model DU, at a wavelength of 510 m $\mu$ .

It is evident from the data in Table I that the livers from vitamin B<sub>12</sub>-deficient rats exhibit a lower ability to form methionine from homocystine and either choline or betaine, as compared with those from animals dosed with vitamin B<sub>12</sub>. Since, according to recent evidence (8,9), it is doubtful that a direct transmethylation from choline occurs, only a deficiency in the transmethylation from betaine would appear to be involved. Furthermore, this supposition is confirmed by the fact that the effects of vitamin B<sub>12</sub> are equally marked when betaine is substituted for choline. A decrease in liver and kidney choline oxidase activity has been reported for folic acid deficiency in monkeys (10). However, lack of folic acid could not be a limiting factor in the present studies, as the diet contained adequate folic acid (200  $\mu$ g./100 g.), and no decrease in choline oxidase was observed in these livers.

Attempts at *in vitro* activation of the methionine-forming system by addition of solutions of crystalline vitamin B<sub>12</sub> Merck have thus far been unsuccessful, but the data presented are sufficient to demonstrate that, under the conditions employed, vitamin B<sub>12</sub> is involved in either the formation or the activation of the methionine forming system.

We are indebted to Dr. Gladys A. Emerson for the rats used in these studies.

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## **Erratum**

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**In Table I, the column headed, "Urea counts/min./mg.,"  
should read, "Urea counts/min. 'mmole."**

# Inactivation Studies on Cholesterol Esterase

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## INTRODUCTION

Pancreas homogenate fractions can be differentially inactivated towards monovalent alcohol esters on the one hand and glycerol esters on the other by pretreatment of the enzymes with heat, alkali, or crystalline trypsin (1). The enzymatic behavior towards cholesterol esters is of particular interest owing to the paucity of information about cholesterol esterase in general. The aim of this investigation is to establish whether, by similar criteria, cholesterol esterase can be distinguished from the enzyme or enzymes which act upon the previously investigated monovalent aliphatic esters or, whether in this instance we are dealing with a specific esterase. In addition, inactivation studies on the synthetic activity of cholesterol esterase have been made in certain instances.

The procedures have included the following: (a) Establishment of the optimal range of pH for both hydrolysis and synthesis of a cholesterol ester, and (b) the effect on cholesterol esterase activity of heat inactivation, of alkali inactivation, and of inactivation by treatment of the pancreas homogenates with crystalline trypsin.

## EXPERIMENTAL

### *The Substrate*

Cholesterol butyrate was chosen in order to effect a comparison with those aliphatic butyric acid esters investigated. The ester was prepared by adding 1.5 moles of butrylchloride at a slow rate to a chilled solution of cholesterol in an excess of pyridine. The reaction mixture was then poured into an excess of ice-cold dilute sulfuric acid. The semi-solid, nonaqueous lower layer was taken up in ether, neu-

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tralized with bicarbonate solution, and evaporated to dryness. The crude ester was recrystallized three times by dissolving in ether and precipitation with absolute alcohol at  $-20^{\circ}\text{C}$ . (m.p.  $97^{\circ}\text{C}$ ., uncorr.).

*Analysis.* Calcd.: C, 81.5; H, 11.5. Found: C, 81.5; H, 11.5.

Stable emulsions of the ester were obtained by dissolving fractions of a millimole in a few milliliters of hot acetone and pouring this solution into 20 ml. of an aqueous 2% solution of ox bile powder (Merck).<sup>3</sup> The emulsion was then evaporated to half its volume, and an equal amount of 0.2 *M* phosphate buffer was added. While no hydrolysis of the ester occurs in the presence of the buffer, about 2% of the ester is hydrolyzed in the course of the above evaporation. The amounts of free cholesterol and of total cholesterol were therefore determined in each sample of the ester emulsions. The stability of these emulsions at  $38^{\circ}\text{C}$ . was examined, and constant values were obtained in aliquots removed in the course of 3 hr. Incubation with the enzyme was never extended over 1 hr. The same emulsification procedure was employed with cholesterol when the enzymatic synthesis was investigated.<sup>3</sup> An equivalent amount of butyric acid was then added to the emulsion.

### *The Enzyme*

Hog pancreas homogenates were used as in the previous study (1). Dialysis of these homogenates at  $5^{\circ}\text{C}$ . did not reduce their activity compared to the nondialyzed control, nor did alcohol fractionation or differential centrifugation at  $26,000 \times g$  bring about a marked change in activity in either pellet or supernatant liquid. The homogenates were therefore used without being subjected to any further treatment.<sup>4</sup> Cholesterol blanks were run for all the homogenates.

### *Method of Assay*

The method of Schoenheimer and Sperry has been used with small modifications as introduced later (2, 3). A Beckman spectrophotometer was employed.

### *The pH Curve*

Phosphate buffer mixtures were used over the range between 5.2 to 8. To obtain a pH lower than 5 without changing the basic composition of the buffer, varying amounts of 5% HCl were added to buffer mixtures of pH 5.2. The pH of the enzyme-substrate mixture was checked at the beginning and at the end of the incubation with a glass electrode. In the ester synthesis experiments the pH of the cholesterol-butyric acid-enzyme mixture remained constant throughout the whole incubation time owing to the strong buffering capacity of the rather concentrated homogenates used, and to the fact that the excess of nonesterified butyric acid (about 70%)

<sup>3</sup> Emulsions prepared with either lithocholic acid or neutralized solutions of lithocholic acid were comparatively unstable.

<sup>3</sup> Cholesterol (Eastman Kodak & Co.) was twice recrystallized from ether.

<sup>4</sup> The ratio between the activity of the supernatant liquid and the activity of the resuspended pellet was 0.76, both for the aliphatic butyric acid esters and for cholesterol butyrate.

sufficed to keep the pH at its former value: yet, a small fluctuation of about 0.2 pH unit was observed in the course of the hydrolysis. Values of pH in this instance refer therefore to the initial pH. The results are given in Fig. 1.

### Heat Inactivation

Heat inactivation was carried out as described before (1). The pH of the enzyme-substrate mixture was adjusted to 6.3 (hydrolysis). The results are given in Table I.

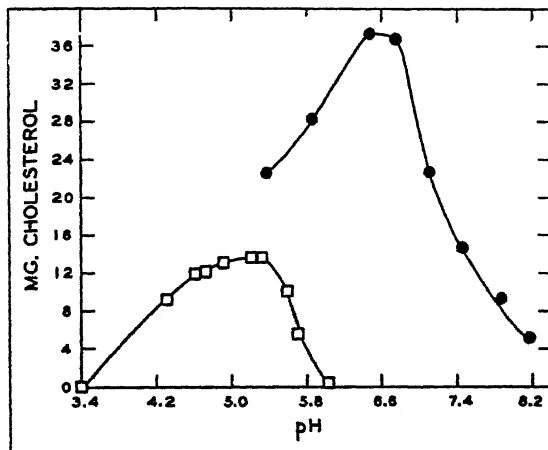


FIG. 1. Range of optimal hydrogen-ion concentration for hydrolysis and synthesis of cholesterol butyrate. Concentration of cholesterol butyrate: 122  $\mu$ moles in 10 ml. of buffer-ox gall extract mixture. Concentration of cholesterol and butyric acid: 51  $\mu$ moles in 10 ml. of buffer-ox gall mixture. To 10 ml. of the ester emulsion 3 ml. of homogenate was added containing 0.844 mg. N/ml. To 10 ml. of the cholesterol emulsion, 3 ml. of homogenate was added containing 7.4 mg. N/ml. Incubation time, 60 min. Values for synthesis are multiplied by 2. ● Hydrolysis. □ Synthesis.

### Inactivation by Pretreatment with Alkali

Owing to the fact that concentrated homogenates have to be used for the study of ester synthesis, the previous procedure had to be modified. One part of hog pancreas was homogenized with one part of distilled water. Small amounts of 2 or 2.5 N NaOH were added dropwise. At the end of 25 min., a previously established amount of 10% acetic acid was added with strong shaking which uniformly reduced the pH to between 5.3–5.8. Equal parts of these pretreated homogenates and of phosphate buffer were then united, and 3 ml. was added to 10 ml. of cholesterol-butyric acid emulsion brought to the same pH. Determinations of pH revealed no changes during the course of the incubation. When the hydrolysis of the ester was investigated the pretreated homogenates were used in a 15-fold higher dilution. The results are given in Fig. 2.



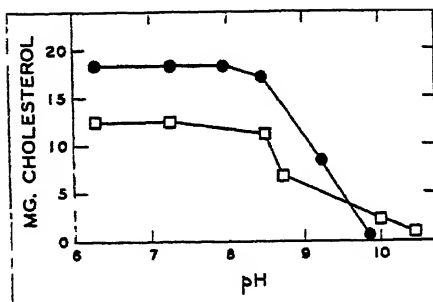


FIG. 2. Alkali inactivation. Numbers on the abscissa indicate pH at which the homogenates were pretreated for 25 min. Concentration of cholesterol butyrate: 88  $\mu$ moles in 10 ml. of emulsion. Concentration of cholesterol and butyric acid: 92 and 100  $\mu$ moles, respectively. Concentration of the homogenates: 5.08 mg. N/ml. for ester synthesis, 0.374 mg. N/ml. for hydrolysis. Enzyme-substrate mixtures as in previous tables. ● Hydrolysis. □ Synthesis.

#### *Pretreatment of Pancreas with Crystalline Trypsin*

Fraction  $P_L$ , isolated from trypsin treated hog pancreas homogenates (1), failed to hydrolyze cholesterol butyrate at a measurable rate of activity or to bring about its synthesis from its components.

#### DISCUSSION

As shown in Fig. 1 there is a marked difference between the optimal pH requirements of the ester hydrolysis on the one hand and its enzymatic synthesis on the other. At the hydrolysis optimum of pH 6.3, the synthetic activity is practically nil. The optimum for the hydrolysis is in agreement with that found for commercial pancreatin by Yamamoto, Goldstein, and Treadwell using cholesterol oleate as a substrate, but differs considerably from the data given by W. Klein for beef pancreas (4, 5).

Since there is no apparent ester synthesis at pH 6.3, there is little likelihood of an equilibrium at this pH between ester synthesis and hydrolysis, as has been found with respect to other esters (6), although at a lower pH value a range exists where the reaction can proceed in either direction.

The slopes of the heat inactivation curves of pancreas homogenates show a sharp drop for methyl butyrate in the 50–55°C. interval (1). The drop in the initial activities towards cholesterol butyrate under the same conditions is somewhat smaller (Table I). However, inactivation

by heating to 60°C. reduces the enzymatic activity to a small fraction of its former value for both substrates.<sup>5</sup> Except for a generally reduced activity, incubation at pH 6 does not change the slope of the alkali inactivation curve if cholesterol butyrate is substituted by methyl butyrate. The activity of the homogenate towards cholesterol butyrate is not changed by pretreatment to pH 5 for 2 hr., while the alkali inactivation curves for both synthesis and hydrolysis are similar (Fig. 2). Pretreatment of pancreas homogenates with crystalline trypsin reduced both activities practically to zero.

TABLE I  
*Heat Inactivation of Glycerolated Pancreatic  
Homogenates at Various Temperatures<sup>a</sup>*

To 10 ml. of buffer-ox gall mixture containing 14 mg. cholesterol butyrate was added 3 ml. of pretreated enzyme containing 0.504 mg. of nitrogen. Activities were estimated from the linear parts of the time curves.

Temperature (°C.)	38	50	55	60	65
Initial rate of activity ( $\mu$ moles/ mg. N/hr.	50	42	22	<1	0.00

<sup>a</sup> All samples pretreated for 25 min. at indicated temperatures.

Comparison of the enzymatic behavior of cholesterol butyrate with that of its aliphatic monovalent alcohol ester analogs (1) reveals a striking similarity with respect to all the aforementioned inactivation procedures. With the exception of the difference in the pH optima, which for all aliphatic esters and also for benzyl butyrate are on the alkaline side, no basic difference in the enzymatic susceptibility of cholesterol butyrate was detected. It is thus probable that both cholesterol esterase and the esterases which hydrolyze methyl butyrate and its homologs are either very similar to each other or else identical.

### SUMMARY

The pH optima for the hydrolysis and synthesis of cholesterol butyrate by hog pancreas homogenates are, respectively, 6.3 and 5.2

<sup>5</sup> The activities of nonheated homogenates towards cholesterol butyrate and methyl butyrate are, respectively, 150 and 1016, and of homogenates heated to 60°C., 0 and 68, expressed in  $\mu$ moles/mg. of enzyme N/hr. In this comparative experiment the concentration of methyl butyrate and cholesterol butyrate was, respectively, 1 mmole and 0.1 mmole in 13 ml. of enzyme-substrate mixture.

under the experimental conditions. The activity of these homogenates is reduced nearly to zero by heat treatment at 60°C., alkali treatment to pH 10, and treatment with crystalline trypsin.

With the exception of the difference in the pH optima no further difference in the enzymatic behavior of these pretreated homogenates was found towards monovalent aliphatic alcohol esters on the one hand and towards cholesterol butyrate on the other.

The possible identity of cholesterol esterase in pancreas with other pancreatic esterases has been discussed.

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## Folic Acid-Like Activity from Xanthopterin

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### INTRODUCTION

Xanthopterin (2-amino-4,6-dihydroxypteridine) is notable for the lack of reproducibility of its biological activities (1). In a previous paper (2) attention was directed to the occurrence of purine-like impurities in certain samples of xanthopterin, which appeared to be pure as judged by chemical and physical criteria. These impurities could be eliminated by modifications in the method of preparation. The present communication describes a folic acid-like activity which appears when xanthopterin solutions containing sugars or sugar derivatives are autoclaved and subsequently tested in the presence of a source of *p*-aminobenzoylglutamic acid. This microbiological activity appears to be the end result of two processes: First, a chemical reaction to produce small quantities of some as yet unidentified pteridine precursor, and secondly, the utilization of this derivative with *p*-aminobenzoylglutamic acid either in lieu of or in the actual synthesis of folic acid by the micro-organism.

### EXPERIMENTAL

#### *Medium*

The medium and the culture of *Lactobacillus casei* have been described in an earlier paper (3). The O medium, used in the present studies, is essentially the medium of Landy and Dicken (4) with omission of purines and pyrimidines. The medium for *Streptococcus faecalis* R was essentially that of Luckey *et al.* (5) with omission of purines and pyrimidines. Growth was measured nephelometrically using a photo-electric colorimeter.

### RESULTS

In the course of routine studies of pteridines as possible antagonists of nucleic acid derivatives (3), xanthopterin was tested in the usual

way (3) and in the presence of *p*-aminobenzoylglutamic acid (*p*ABG). Xanthopterin was found to be unique among the pteridine derivatives in that it shows considerable folic acid-like activity when *p*ABG is also present (Table I). However, when xanthopterin and *p*ABG were added aseptically to the previously autoclaved medium the activity did not appear. Thus it was recognized that an active substance is formed by the heating of xanthopterin with *p*ABG and/or some constituent of the medium. When a mixture of xanthopterin and *p*ABG was autoclaved with glucose and added aseptically to the sterile medium, growth was supported. Of binary combinations of the three substances, activity results only from the heating of xanthopterin with glucose.

TABLE I  
*Effect of Pteridines on the Growth of Lactobacillus casei in the Presence of p-Aminobenzoylglutamic Acid*<sup>a</sup>

Compound	Acid production ml. of 0.1 N/10 ml.
Xanthopterin	12.5
7-Methylxanthopterin	0.7
Isoxanthopterin	0.6
6-Methylisoxanthopterin	0.6
Xanthopterin-7-carboxylic acid	1.0
Isoxanthopterin-6-carboxylic acid	0.6
$\alpha$ -Dihydroxanthopterin	0.8
$\beta$ -Dihydroxanthopterin	0.8
Leucopterin	0.8
2-Amino-6-hydroxypteridine	0.8

<sup>a</sup> One mg. of the pteridine and 5 mg. of *p*ABG were added to 10 ml. of the O medium (3) and autoclaved.

The specificity of glucose in this action was tested by heating xanthopterin with other sugars and related substances. Several of the products were able to support growth in the presence of *p*ABG as is shown by the data of Table II. From these data it appears that an available carbonyl group and a chain length of at least three carbon atoms are required for the production of the active substance.

The data showing the effects of various proportions of xanthopterin and *p*ABG when autoclaved in the medium are given in Fig. 1. In this graph, the growth of *Lactobacillus casei*, as measured by acid production is plotted against the concentration of xanthopterin for each of three concentrations of *p*ABG. Similar data were obtained using *Streptococcus faecalis* R.

TABLE II

*Production of Folic Acid-Like Activity by Heating Xanthopterin with Various Substances<sup>a</sup>*

Substance	Acid production ml. 0.1 N 10 ml.	Substance	Acid production ml. 0.1 N 10 ml.
D-Glucose	12.8	Formaldehyde	0.7
L-Arabinose	12.7	Acetaldehyde	1.6
D-Fructose	12.1	D-Sucrose	1.9
D-Ribose	12.2	Glycerol	2.3
Dihydroxyacetone	13.8	Control	1.3

Xanthopterin (10 mg.) was dissolved in 10 ml. of water with 1 equivalent of sodium hydroxide solution; the indicated compound (50 mg.) was added and the solution was heated at 15 lb. pressure for 20 min. A 1-ml. portion of the cooled solution was added aseptically to 10 ml. of O medium (3) containing 5 mg. of *p*-aminobenzoylglutamic acid.

Since the folic acid requirement of *S. faecalis* R. can be satisfied equally well by pteric acid and by folic acid (1) whereas *L. casei* responds only to the glutamic acid derivatives (1) it was of interest to determine the response of these organisms to the xanthopterin-glucose product when *p*-aminobenzoic acid (*p*AB) was substituted for *p*ABG. No response was obtained with *L. casei* but, in line with expectation,

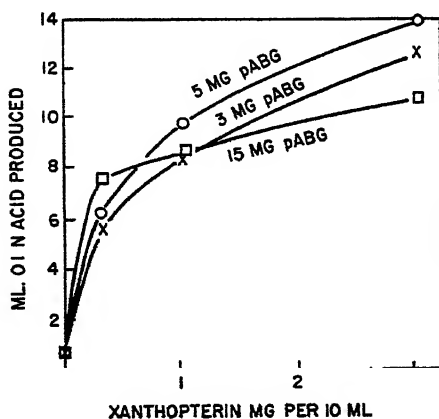


FIG. 1. Growth of *Lactobacillus casei* with xanthopterin-glucose product and *p*-aminobenzoylglutamic acid as a substitute for folic acid. The indicated quantities of xanthopterin and *p*-aminobenzoylglutamic acid (*p*ABG) solutions were added to 10 ml. of medium, and the medium subsequently was heated in the autoclave (15 lb., 20 min.)

*S. faecalis* responds essentially as well to *p*AB as to *p*ABG. This is shown in Fig. 2 where the growth response (optical density) is plotted against the xanthopterin concentration for each of 3 levels of *p*AB. It is to be noted that for the higher levels of *p*AB a maximum response is obtained with 0.4 mg. of original xanthopterin/10 ml., and at 1.0 mg. of xanthopterin when *p*ABG is used. The ratio of the components for maximal response is thus different for the two organisms, since *L. casei* (Fig. 1) appears to require a considerably higher proportion of the xanthopterin reactant for maximal response than does *S. faecalis* R.

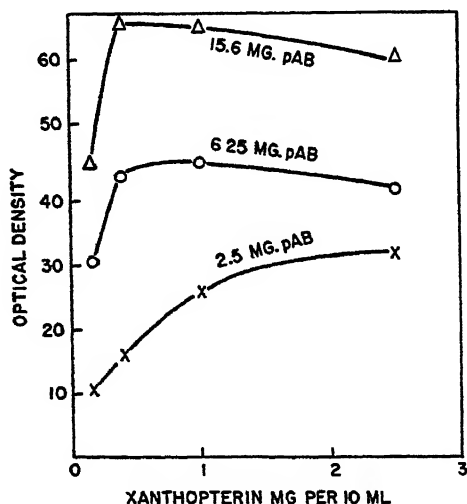


FIG. 2. Growth of *Streptococcus faecalis* R. with xanthopterin-glucose product and *p*-aminobenzoic acid as a substitute for folic acid. The indicated quantities of xanthopterin and *p*-aminobenzoic acid (*p*AB) solutions were added to 10 ml. of medium, and the medium was subsequently heated in the autoclave (15 lb., 20 min.).

In order to test the nature of the stimulatory substance formed from xanthopterin, a study was undertaken of the effects of various inhibitors on growth with this substance, as compared with folic acid. Control determinations showed that neither xanthopterin nor  $\alpha$ -dihydroxanthopterin interferes with the activity of the inhibitors in the folic acid medium. It will be seen that in most instances reasonably good agreement exists between the effects of a given inhibitor on the two growth systems (Table III). However, some rather outstanding and significant differences exist. Thus 5-hydroxyuracil (Expt. 2) fails to give more than

minimal inhibition. Since reversal of the inhibitory effects of this substance does not take place with folic acid or purine but only with uracil (3) this would appear to indicate either that the essential nutritive in the xanthopterin system differs from folic acid or that a source of uracil is present in the medium. Similarly, the results with 5-bromouracil (Expt. 5) and with diaminopurine (Expt. 8) indicate either that a source of purine is present in the medium or that the two nutritives differ fundamentally. No purine-like activity could be detected by

TABLE III

*Comparison of Effects of Antagonists on Growth of L. casei with Two Nutritives*

No.	Substance	Concentration	Acid production	
			Xanthopterin <sup>a</sup>	Folic acid <sup>b</sup>
		mg./10 ml.	ml. 0.1 N 10 ml.	ml. 0.1 N 10 ml.
1	Control	—	6.7	6.0
2	5-Hydroxyuracil	1.0	5.0	0.5
3	5-Aminouracil	1.0	1.5	1.0
4	5-Nitrouracil	1.0	1.0	0.5
5	5-Bromouracil	1.0	7.2	4.8
6	2,4-Diamino-5,6-dimethylpyrimidine	1.0	0.9	0.2
7	2,4-Diamino-5- <i>p</i> -chlorophenoxy- pyrimidine	0.01	1.0	0.2
8	2,6-Diaminopurine	0.05	8.4	0.9
9	2,4-Diamino-6,7-dimethylpteridine	0.05	2.0	3.0

<sup>a</sup> Product of heating 1 mg. xanthopterin with 5 mg. of glucose tested in the presence of 5 mg. of *p*ABG in 10 ml. of medium.

<sup>b</sup> 0.45  $\mu$ g./10 ml. Cf. Hitchings *et al.*, *J. Biol. Chem.* **183**, 1 (1950); **174**, 765 (1948).

direct test in thymine medium. However, the interpretation of these results is complicated by the fact that xanthopterin in itself has inhibitory effects (2). A final selection between the two alternatives, therefore, must await the purification of the xanthopterin-glucose product.

## DISCUSSION

The product formed by heating xanthopterin with glucose in aqueous solution has biological properties quite different from those of xanthopterin itself. This observation highlights a possible source of error and misinterpretation with respect to the biological activities of xanthop-



terin and may account for some of the discrepancies and irregularities which are recorded in the literature.

The growth of *L. casei* with the xanthopterin-glucose product and pABG indicates the possibility that this microorganism may be able to perform a partial synthesis of folic acid. However, two other interpretations should not be overlooked at this time. It is conceivable that a combination of a more or less specific pteridine derivative and pABG may be able to function in lieu of folic acid without actual chemical union. It is possible also that a folic acid-like substance differing in some respects from folic acid may be formed and function as folic acid. Either of the latter interpretations would account for the differences which are observed in the response to inhibitors between the folic acid-grown and xanthopterin-grown organisms.

#### SUMMARY

When xanthopterin is heated in aqueous solution with glucose a product is formed which, with *p*-aminobenzoylglutamic acid (pABG) can supply the folic acid requirement of *Lactobacillus casei* or *Streptococcus faecalis* R. It cannot be decided on the basis of the available evidence whether or not the microorganism synthesizes folic acid under the described conditions. However, some differences in the response to inhibitory substances have been observed in organisms grown with xanthopterin-glucose-pABG as compared with those grown with folic acid.

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# Proteolytic Activity of Normal and Ischemic Kidneys of the Rabbit

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## INTRODUCTION

In order to understand better the activity of the kidney in some pathological conditions, a study of the proteolytic enzymes of the kidney was performed. It is known that cathepsin is the main proteinase present in animal cells and that it is very active in those tissues which synthesize proteins.

This paper deals with cathepsin activity of kidneys of normal rabbits and of kidneys of rabbits in which the blood pressure was artificially increased by means of wrapping silk around the kidneys. A method suitable for the investigation of relatively small amounts of tissue was developed.

## METHOD

The enzyme solution was prepared following Bergmann's modification of Anson's method (1,2). A few changes were introduced in order to make this method suitable for the investigation of relatively small amounts of tissue.

After the third precipitation with ammonium sulfate, instead of allowing the precipitate to settle, centrifugation for 1 hr. at 3000 r.p.m. was found sufficient to separate a precipitate from the supernatant which was siphoned off. The precipitate was redissolved in a quantity of ice-cold redistilled water equivalent to the original weight of the renal tissue. The clear light-brown solution was dialyzed for 3 days in the refrigerator at 5°C. against a 1% NaCl solution; each day the 1% NaCl solution was changed. (It was found, in fact, that such prolonged dialysis was necessary in order to remove all ammonium sulfate from the solution. In all probability, this time might be considerably reduced if shaking dialysis were employed.) Once the dialysis was completed, the activity of the enzyme solution on the hemoglobin substrate could be measured.

### *Preparation of the Hemoglobin Substrate*

In our experiments, the hemoglobin substrate solution was prepared fresh for each determination from hemoglobin powder, obtained from washed-beef red cells, and prepared by the Armour Laboratory.

In a large test tube, 5 ml. of the hemoglobin solution substrate was prepared by adding with an automatic pipet to 4 ml. of a 2.5% hemoglobin solution (containing 2 mg. of merthiolate Lilly 100 ml.), 1 ml. of a solution made 1.35 *M* with respect to acetic acid, and 0.02 *M* with respect to ammonium sulfate. The final pH was 3.5.

### *Estimation of Cathepsin Activity*

Estimation of cathepsin activity was determined in a constant-temperature bath at 36.8°C., according to the technique described by Anson (1). The amount of tyrosine-tryptophan liberated from hemoglobin was measured colorimetrically, using a Klett-Summerson photoelectric colorimeter (with a blue filter No. 42).

As a rule, all assays of enzymatic activity were made in duplicate, and two colorimeter samples were made for each sample. It was found that the difference between the four specimens was negligible.

### *Calibration Curve*

A calibration curve was plotted by adding to 5 ml. of digestion filtrate, 1 ml. of tyrosine solution, 10 ml. of 0.5 *N* NaOH solution and 3 ml. of phenol reagent. Three samples were usually treated in this way, the tyrosine solutions containing 0.075, 0.150, and 0.225 mg., respectively, of L-tyrosine. The values, read on the colorimeter, fell in a straight line. It was possible, in this way to determine how much tyrosine corresponded to each colorimeter reading.

### *Calculation*

The amount of tyrosine liberated by 1 ml. of enzyme solution was converted into milliequivalents (meq.) of tyrosine (0.15 mg. tyrosine = 0.00083 meq. tyrosine), and multiplied by 16 '5 to give the tyrosine milliequivalents of the whole 16 ml. of filtrate. This last figure was finally multiplied by the volume in milliliters of the enzyme solution after dialysis, and divided by the weight in grams of the renal tissue used in the experiment and by the time, in minutes, of enzymic digestion. The resulting figure is expressed as  $\frac{\text{Cu(Hb)} \times 10^4}{\text{min.} \times \text{g.}}$ .

### *Nitrogen Content of the Enzyme Solution*

The amount of total nitrogen in 1 ml. of enzyme solution was estimated in duplicate by the Kjeldahl method.

## PRELIMINARY OBSERVATIONS

In a series of preliminary experiments, it was found that the amount of enzyme is proportional to the amount of tyrosine liberated from the substrate (Fig. 1), and that for a period of 1 hr. digestion, there is approximately a linear relationship between time and enzymic activity (Fig. 2).

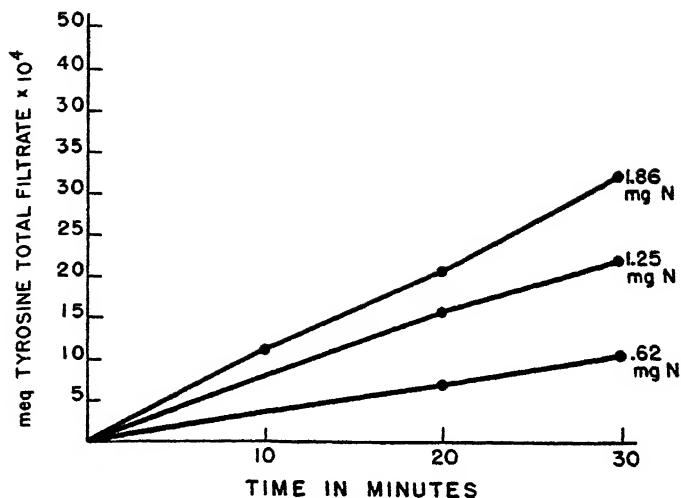


FIG. 1. Catheptic activity curve of three samples of the same enzyme at different concentrations. At right, mg. total nitrogen/ml. of enzyme solution.

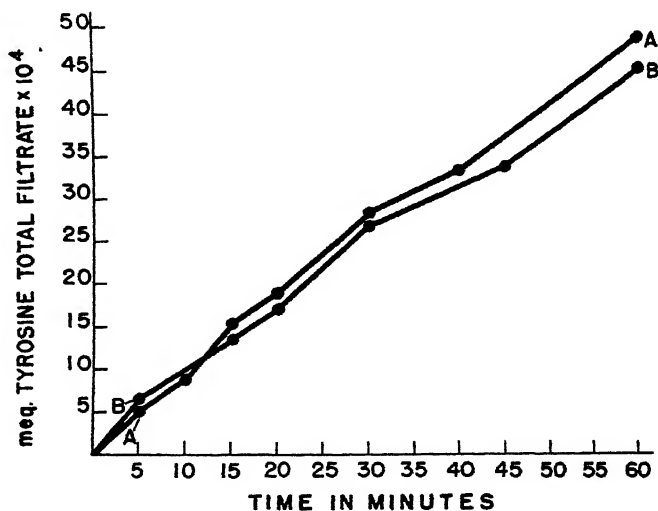


FIG. 2. Catheptic activity of two different enzymic preparations measured for a period of 1 hr. Enzyme A contains 1.130 mg. total nitrogen/ml.; Enzyme B, 0.940 mg. total nitrogen ml.

Enzyme solutions, kept in the refrigerator, slowly lose their activity. These findings confirm previous observations on catheptic activity, made by other investigators.

### *Effect of Cysteine*

In order to investigate the effect of cysteine on our catheptic enzymes, digestions with and without cysteine were performed. In some experiments the difference in tyrosine color after addition of 37% formalde-

TABLE I  
*Effect of Cysteine on Catheptic Activity*

Rabbit no.	Total nitrogen/ ml. enzyme	Digestion time	No cysteine	With cysteine	Increase
	<i>g.</i>	<i>min.</i>	<i>Mg. N in 0.2 ml. filtrate</i>		<i>%</i>
25	0.340	30	0.170	0.186	+ 9.0
		60	0.284	0.346	+21.8
52	0.125	60	0.168	0.236	+40.5
55	0.402	10	0.036	0.044	+22.2
		20	0.070	0.096	+37.1
		30	0.092	0.144	+56.4

Color value: 5 ml. trichloroacetic filtrate plus 1 ml. formaldehyde

			<i>Mg. tyrosine</i>		
NS7	0.129	10	0.0316	0.0457	+44.6
		20	0.0339	0.0574	+71.9
		30	0.0457	0.0785	+71.7
PK4	0.220	10	0.0304	0.0398	+30.9
		20	0.0503	0.0667	+32.0
		30	0.0808	0.1066	+31.9

hyde was noted; in others the amount of nitrogen in the trichloroacetic acid filtrate was determined by the Kjeldahl method on double specimens. A similar technique was used by Anson for his observations (1,3).

In our experiments cysteine seems to have a slight activating effect on cathepsin. Our values range from 9.0 to 71.9% increase of catheptic activity.

A slight cysteine effect (about 20% increase) was obtained by Anson (3) when final normality of trichloroacetic acid was 0.2; Maver instead (4), in some of her samples, noted an increase up to 280%, due to cys-

teine. Schäffner and Truelle (5), and later Lang and Wegner (6), found that cathepsin I can be separated into one enzyme acting on hemoglobin, and into one acting on gelatin. Purified extracts of cathepsin I seem to be activated by cysteine when this effect is studied by determination of the total nitrogen, and not to be activated by cysteine when the tyrosine color is studied. Lang and Wegner suggest the name of cathepsin T for their purified cathepsin which is not activated by cysteine when the tyrosine color method is employed.

As in our experiments the final product is not a purified enzyme, it seems very probable that the observed effect of cysteine is due to the presence in our enzyme of some contaminant.

### EXPERIMENTAL

Determinations of catheptic activity were performed in a series of 7 normal rabbits, and in a series of 5 rabbits made hypertensive by previous wrapping of silk around both kidneys.

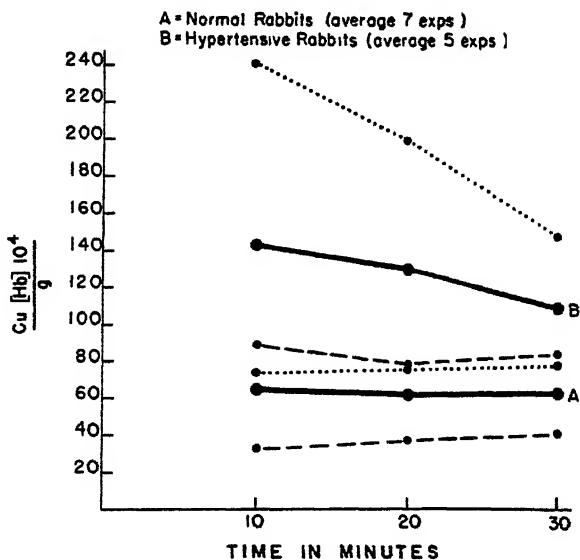


FIG. 3. Catheptic activity, expressed as  $\frac{\text{Cu(Hb)} \times 10^4}{\text{min.} \times g.}$  of normal and hypertensive rabbits. Unbroken lines = average of catheptic units for normal rabbits (A) and hypertensive rabbits (B). Broken lines: ---, scatter of values of normal rabbits, . . . , scatter of values for hypertensive rabbits.

TABLE II  
*Renal (nephritic Activity of Normal and Hypertensive Rabbits*  
 No cysteine added.

Labrat no.	Weight	Weight of kidneys	Weight N/ ml. of em- sine	Tyrosine total fil- trate/10 <sup>4</sup> (after 10 min.)	$\frac{\text{Cu(IIb)} \times 10^4}{\text{mm. Xg.}}$	Tyrosine total fil- trate/10 <sup>4</sup> (after 20 min.)	$\frac{\text{Cu(Hb)} \times 10^4}{\text{mm. Xg.}}$	Tyrosine total fil- trate/10 <sup>4</sup> (after 30 min.)	$\frac{\text{Cu(IIIb)} \times 10^4}{\text{min. Xg.}}$	Mean arterial blood pressure	
Normal rabbits											
PK1	—	30.87	.350	meq. 7.46	0.074	meq. 13.6	0.068	meq. 21.5	0.071	Mean arterial blood pressure of this group has al- ways been below 90 mm. Hg	
NS1	3.14	16.49	.340	7.47	0.074	14.3	0.071	19.5	0.064		
NS2	2.05	13.46	.224	4.55	0.044	9.5	0.047	14.0	0.047		
NS5	2.48	16.12	.378	8.92	0.089	15.7	0.079	25.4	0.084		
NS6	2.45	17.38	.423	8.71	0.087	15.5	0.077	22.1	0.074		
NS7	3.07	20.83	.129	5.59	0.056	11.6	0.058	18.0	0.060		
PK4	—	25.84	.220	3.31	0.033	7.4	0.037	12.4	0.041		
Average					0.070		0.066		0.066		
Hypertensive rabbits											
25	2.37	19.03	.340	23.6	0.241	40.0	0.199	51.5	0.098	Before operat. mm. Hg 70	Before death mm. Hg 110
63	3.83	17.03	.125	8.3	0.074	19.7	0.098	31.7	0.105	95	140
52	2.30	16.19	.310	13.0	0.130	25.1	0.125	24.6	0.115	90	135
55	2.85	16.31	.402	18.4	0.185	30.5	0.154	43.9	0.148	100	140
99	2.12	11.49	.106	7.8	0.078	15.3	0.076	13.8	0.079	95	165
Average					0.141		0.130		0.109		

The animals were kept on a uniform diet and the mean blood pressure was determined at regular intervals, before and after the operation on the kidneys, by means of insertion of a No. 20 needle in the median artery of the ear. The needle was connected to the usual mercury manometer.

The operation was aseptically performed on both kidneys on the same day; the rabbit was anesthetized locally with Procaine solution (10%). The animals were killed four weeks after the operation on their kidneys, and used for the experiments only if it was found that no other apparent disease, beside their hypertension, was present. The lapse of time of 4 weeks was chosen in order to have a uniform and comparable duration of hypertension, and because it has been found that after 4 weeks there is usually a significant increase in blood pressure.

When the animals had to be sacrificed, Nembutal was given intravenously (0.45 ml. kg.). For each animal, histological examination of the kidneys was performed.

### DISCUSSION

It has been thought in the past that renin, a proteolytic enzyme, might belong to one of the four known cathepsins. Even though renin preparations may show some proteolytic activity (7), it was demonstrated that it is not identical with any of the known cathepsins (8). To our knowledge, studies on catheptic activity of ischemic kidneys have never been reported.

Our investigation shows that more cathepsin is obtained from an aqueous extract of ischemic kidneys from hypertensive rabbits than from a similar extract of normal kidney. This proteolytic activity is probably mainly due to cathepsin I.

Histological examination of our material makes evident that necrotic lesions are not present in the series of kidneys obtained from the hypertensive rabbits. Necrotic kidneys of rabbit have been found to possess a lower catheptic activity (9).

It has been suggested by some investigators (10,11) that diminution of oxidative agents is a favorable condition to proteolysis; according to Lee and Chen (12), proteolysis is in inverse ratio to oxygen tension. More recently, Irving, Fruton, and Bergmann (13) studied aerobic and anaerobic proteolysis using synthetic substrata; they showed that proteolysis of carbobenzoxy-L-glutamyl-L-tyrosine by swine kidney pepsinase was identical under aerobic and anaerobic conditions. On the



other hand, proteolysis of cathepsins which are proteolytically active only in the presence of an activator, was significantly higher under anaerobic conditions.

#### ACKNOWLEDGMENTS

I am grateful to Dr. William Antopol for the reading of the histological specimens, to Drs. Erwin Haas and Sarah Ratner for their criticisms, and to Drs. Nahoum J. Winer, Joseph Rechtschaffen, and W. A. Schloss for assisting me in the operations of the rabbits.

#### SUMMARY

More cathepsin is obtained from an aqueous extract of ischemic kidneys from hypertensive rabbits than from a similar extract of normal kidney.

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# Further Studies on the Factors Which Affect the Lipide Oxidation in Rat Liver as Estimated by the Thiobarbituric Acid Test

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## INTRODUCTION

The thiobarbituric acid test on tissues which have been incubated aerobically *in vitro* is primarily a measure of the amount of oxidized linolenic acid which may be present in free or combined form (1). The oxidation of the linolenic acid is catalyzed by ascorbic acid; that this catalysis occurs in most tissues is indicated by the fact that the oxidation is diminished when animals are scorbutic (2). In the liver, however, scurvy does not affect the oxidation significantly, so it was possible that other systems involved in fatty acid oxidation form products which will react with thiobarbituric acid. The factors which affect the lipide oxidation in liver as measured by the thiobarbituric acid test were therefore investigated in more detail and compared with those found necessary for fatty acid metabolism by the system described by Lehninger (3,4). The results showed that the latter was not involved in the oxidation of linolenic acid as estimated by thiobarbituric acid.

## EXPERIMENTAL

Rat-liver suspensions were made by grinding in a mortar and straining through cheesecloth. Twenty mg. (wet weight) of tissue was incubated aerobically in 4.0 ml. of various buffers at 37° for 3 hr. The protein was then precipitated with 1.0 ml. 20% trichloroacetic acid, and the thiobarbituric acid added to the clear centrifugate and the color developed and estimated by the method previously described (1).

### *The Effect of pH, Ascorbic Acid, ATP and Cytochrome c*

Kohn and Liversedge (5) showed that the maximum thiobarbituric acid color was produced when tissues were incubated between pH 5.6

and 6.0. This in itself would differentiate the oxidation from that catalyzed by Lehninger's system which is active only between pH 7.0 and 8.0. It was possible, however, that the pH effect might be due to the destruction of accessory factors. Therefore  $2 \times 10^{-3} M$  adenosinetriphosphoric acid (ATP) and  $1 \times 10^{-5} M$  cytochrome c were added alone or together with and without  $2.8 \times 10^{-4} M$  ascorbic acid at pH 6.0 and 7.8. The results, given in Table I, show that neither ATP nor cytochrome added separately or together had any striking effect, but either

TABLE I

*Effect of Ascorbic Acid, ATP and Cytochrome*

The effect of  $2.8 \times 10^{-4} M$  ascorbic acid,  $2 \times 10^{-3} M$  ATP, and  $1 \times 10^{-5} M$  cytochrome c was determined separately and together on the values obtained with the thiobarbituric acid test after incubating 20 mg. of rat liver suspension aerobically for 3 hr. at 37° at pH 6.0 and 7.8. The figures are the color density as measured in the Evelyn photocolormeter and are the average of three experiments.

Additions	Color density at pH 6.0	Color density at pH 7.8
Liver alone	0.258	0.087
+ ascorbic acid	0.398	0.268
+ ATP	0.306	0.098
+ cytochrome c	0.224	0.058
+ cytochrome c+ATP	0.242	0.073
+ ascorbic acid+cytochrome c	0.478	0.356
+ ascorbic acid+ATP	0.455	0.433
+ ascorbic acid+cytochrome c+ATP	0.446	0.392

added with ascorbic acid gave significant increases above the values given by ascorbic acid alone. Similar results were obtained with brain suspensions. Apparently either cytochrome c or ATP can increase the catalytic affect of ascorbic but since this effect can also occur after heating the suspension to inactivate the enzymes, it is obvious that the fatty acid oxidase system is not involved.

*The Effect of Carboxylic Acids*

Lehninger (3) showed that oxalacetic, citric, and other members of the Krebs cycle added to the enzyme system increase the oxidation of the fatty acid. These acids and certain others either markedly inhibit the oxidation of linolenic acid as estimated by the thiobarbituric acid color or are without effect. The results are shown in Table II. Citric acid is the most effective inhibitor followed by oxalic and pyruvic acids in this order. Malic and fumaric acids cause slight inhibitions, and oxalacetic, lactic, and malonic acids are without effect. If citric acid is

added after the oxidation has been allowed to progress almost to completion, no reduction in the thiobarbituric acid color values was obtained which indicates that citric acid did not reverse the reaction once it had occurred. Excess calcium did not overcome the inhibition. This experiment was done in phthalate buffer to prevent the formation of calcium phosphate. The oxidation proceeds in phthalate as well as it does in phosphate, but the latter is necessary for the oxidase system. Magnesium ions which are also a necessary component for the oxidase have no effect on the oxidation either in the presence or absence of ascorbic acid.

TABLE II  
*Carboxylic Acids*

The effect of various carboxylic acids on the values obtained by the thiobarbituric acid test after incubating 20 mg. of rat liver suspension aerobically for 3 hr. at 37° at pH 6.0 was determined.

Carboxylic acid added as sodium salt	Color density
Liver alone	0.417
+0.005 <i>M</i> citrate	0.036
+0.005 <i>M</i> oxalate	0.195
+0.005 <i>M</i> pyruvate	0.280
+0.005 <i>M</i> malate	0.337
+0.005 <i>M</i> fumarate	0.330
+0.01 <i>M</i> oxalacetate	0.408
+0.01 <i>M</i> lactate	0.419
+0.005 <i>M</i> malonate	0.417

In the absence of tissue, citric acid also inhibited the oxidation of  $3 \times 10^{-4}$  *M* pure methyl linolenate or linolenic acid and of phospholipides extracted from brain. The inhibition occurred both in the presence and absence of ascorbic acid. The best inhibitions (40–60%) were obtained when the oxidation was allowed to proceed slowly at room temperature for 1–5 days. Pyruvic acid, on the other hand, increased the oxidation 50–200%. It is possible that the inhibition produced by pyruvic acid on liver suspensions is caused by the formation of citric acid from pyruvate.

#### *The Effect of Metal Salts*

Both ferrous and ferric salts added to liver suspensions increased the oxidation to the same extent as ascorbic acid. The optimal concentration of the iron was  $1.0 \times 10^{-3}$  *M*. Citric acid did not inhibit the catalysis but increased it, possibly because it kept the iron in solution. The mechanism of the iron catalysis is presumably different from that of

ascorbic acid. Copper, cobalt, nickel, manganese, tin, and chromium salts were without effect in the presence or absence of citric acid. Iron salts were better catalysts than ascorbic acid for the oxidation of pure methyl linolenate and here again citric acid increased the effect. Adrenaline which inhibits the ascorbic acid catalysis in liver and of pure methyl linolenate also inhibited the iron catalysis in both cases.

### DISCUSSION

The oxidation of linolenic acid in liver as estimated by the thiobarbituric acid test is catalyzed by ascorbic acid or iron salts. Either cytochrome c or ATP can increase the catalytic effect of ascorbic acid but the mechanism of this is not clear. These two compounds are the only components of the fatty acid oxidase system as described by Lehninger which play some part in the oxidation estimated by the thiobarbituric acid. In all other respects the two systems differ. Phosphate is necessary for the oxidase and certain carboxylic acids act catalytically. For the other system, phosphate is not necessary and the carboxylic acids inhibit. Aging the liver suspension, which destroys DPN, inactivates the oxidase (6) but has no effect on values obtained with thiobarbituric acid. Finally the pH optima of the two oxidations are different and whereas the oxidase requires magnesium the other system does not. It thus seems obvious that there are two separate mechanisms for the oxidation of linolenic acid in liver. The one is the fatty acid oxidase which oxidizes it and other fatty acids; the other which is concerned primarily with the oxidation of linolenic acid results in a product which reacts with thiobarbituric acid.

### SUMMARY

1. Various factors which affect the oxidation of linolenic acid in liver as estimated by the thiobarbituric acid test have been described.
2. The system responsible for this oxidation is different from the fatty acid oxidase as defined by Lehninger and his co-workers.

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# Stabilization of Mitochondrial Activity by Vitrification

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## INTRODUCTION

Several laboratories have reported the extreme lability of the oxidative and phosphorylative activity of mitochondrial suspensions (1-5), the techniques of lyophilization, freezing, and dehydration with cold acetone, *etc.*, resulting in the almost total inactivation of the preparations. Routine study of mitochondrial activity therefore has required the daily preparation of fresh material from living animals, preparations whose  $Q_{O_2}$  and  $Q_{PO_4}$  vary by as much as 100% from day to day. In consequence, the general practice in such work consists in referring all experimental results to a daily standard, rather than to a preparation that remains uniform from day to day.

We have recently conducted some experiments<sup>2</sup> designed to provide a stabilized mitochondrial suspension, and have found that both oxidative and phosphorylative activity may be recovered after rapid freezing at  $-80^{\circ}\text{C}.$ , ("vitrification") (6,7). Present techniques demonstrate a P/O ratio of approximately 1.2.

## METHODS

### *Preparation of Mitochondrial Suspension*

Four kidneys from two rabbits killed by a blow on the head were homogenized in a Waring Blender for 2 min. in 100 ml. cold 30% sucrose-0.02 M  $\text{NaHCO}_3$  and filtered through two layers of clean gauze. This homogenate was then centrifuged at 1200 r.p.m. for 3 min. and the supernatant liquid carefully siphoned off. The supernatant

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<sup>2</sup> A preliminary report of this work was presented before the American Society of Biological Chemists, Detroit, Michigan, April 20, 1949 [Loomis, W. F., *Federation Proc.* 8, 220 (1949)].

TABLE I

Vessels contained 1.0 ml. mitochondrial suspension as described above, to which were added 0.1 ml. 0.2 *M* MgCl<sub>2</sub>; 0.2 ml. 0.01 *M* Na adenosine-5-phosphate pH 7.2, 0.3 ml. 0.1 *M* Na glutamate pH 7.2; 0.3 ml. 0.2 *M* potassium phosphate pH 7.2; with deionized water to make 2.8 ml. For further details see Ref. (4) and (8).

Expt.	Time of storage at - 73°C.	Oxygen uptake <sup>a</sup>	Per cent activity recovered
1	Freshly prepared	170.0	
	Frozen 30 min.	157.7	92
	Frozen 24 hr.	115.0	67
2	Freshly prepared	263.0	
	Frozen 4 days	223.0	85
	Frozen 7 days	158.2	60

<sup>a</sup> Cu. mm. in 10 min. at 37°C.

liquid was then mixed with an equal volume of cold 0.1 *M* tris (hydroxymethyl) amino methane pH 7.2<sup>3</sup> and centrifuged for 30 min. at full speed in a refrigerated centrifuge (3000 r.p.m.). After discarding the supernate, the residue of packed mitochondria was suspended evenly in 16 ml. of cold 9% sucrose-0.01 *M* Na citrate pH 7.2 and 0.04 *M* tris (hydroxymethyl) amino methane pH 7.2.

### *Technique of Vitrification and Thawing*

Five ml. of the above suspension was pipetted into the bottom of a 15-ml. ampoule provided with a drawn-out neck. The ampoule was then closed in a flame and cooled. Holding the neck of the ampoule, the remainder was lowered with a swirling motion into a pan of Dry Ice-alcohol that had previously been allowed to reach approximately - 100°C. The thick yellowish suspension of mitochondria freezes almost instantaneously under these conditions. Storage was carried out under Dry Ice in an insulated box.

Thawing was effected by submersing the frozen ampoule in a Warburg bath at 37°, rotating it rapidly until the last piece of frozen suspension just melted, without the main body of the suspension rising above 5°. The neck of the ampoule was then broken and the preparation tested.

### DISCUSSION

Since even fresh suspensions of mitochondria are unstable at room temperature in the absence of substrate molecules (1) it was suspected that inclusion of citrate in the freezing medium might serve to stabilize the preparation, especially when it was being thawed at 37°. Thus in one experiment it was found that only 26% of the original activity was

<sup>3</sup> Commercial Solvents Corporation, 17 East 42nd Street, New York, N. Y.

recovered in the absence of included citrate, while 83% was recovered when the vitrifying medium was made 0.01 *M* with respect to sodium citrate pH 7.2.

Further experiments demonstrated that activity was best maintained at temperatures lower than those provided by a commercial deep freeze. Such temperatures were provided in these preliminary experiments by the relatively crude method of keeping blocks of Dry Ice in a deep freeze, storing the frozen ampoules in a container to prevent breakage. Even with these less than optimal methods of storage, considerable stability was achieved (Table I) and further development with respect to both the freezing medium and the conditions of storage promise to yield a satisfactory method of providing a standardized preparation that will remain uniform from day to day.

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# Contribution to the Structure of Pro- $\gamma$ -Carotene and Prolycopene Obtained from Various Sources

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## INTRODUCTION

Although preparations of a given carotenoid, originating from various sources, generally have identical physical constants, this is not the case for  $\gamma$ -carotene,  $C_{40}H_{56}$ , as was pointed out earlier in collaboration with W. A. Schroeder (1). Analytically pure samples of the hydrocarbon mentioned showed melting points extending over a range of  $47^\circ$ , viz.,  $131$ – $178^\circ\text{C}$ . Polymorphism may account for some of these deviations, but it is also possible that some of the samples described as  $\gamma$ -carotene are structurally slightly different from the accepted formula. If they did not contain the isolated double bond near the end of the aliphatic terminal group, this fact would not have been revealed either by combustion analyses or by spectroscopic observations.

A re-investigation of the structure of certain samples along these lines became desirable when the strong provitamin A activity of a poly-*cis*  $\gamma$ -carotene, termed pro- $\gamma$ -carotene, was observed in collaboration with Professor Deuel and his group. This *cis* compound showed (both in the rat and in the chick) at least the same potency as did its all-*trans* isomer prepared by iodine catalysis (2,3). Recently, we have submitted various preparations to ozonolysis and subsequent oxidation, as well as to catalytic hydrogenation.

## EXPERIMENTAL

The isopropylidene group estimations were carried out according to Kuhn and Roth (6) (20 ml. of 1.5% ozone, min. was passed through a glacial acetic acid solution, during 2 hr.). After permanganate oxidation and distillation, the acetone was estimated iodometrically.

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<sup>1</sup> Contribution No. 1373.

In order to confirm the identity of the acetone formed, fractions of the distillate obtained from 32.5 mg. of pro- $\gamma$ -carotene were treated with 2,4-dinitrophenylhydrazine. The precipitate collected from the first 10-ml. fraction weighed 6.7 g. (The second and third similar fractions gave only traces of the hydrazone.) The melting point of the crude hydrazone was 121–2°. A chromatographic development on silicic acid with benzene + petroleum ether, b.p. 60–70° (1:1) gave a broad main zone, accompanied by a narrow zone, the latter containing only traces of material. The main zone was eluted with ethylene chloride + petroleum ether (1:1) and the substance was recrystallized from ether. Its m.p., 125–6°, was not depressed by an authentic sample of acetone dinitrophenylhydrazone. The amount of acetone calculated from the recovered phenylhydrazone (after a suitable correction for solubility) represents 88% of that determined by the iodometric method. The corresponding figure for ozonized and oxidized  $\beta$ -carotene (109.8 mg.) was found to be 97%.

The hydrogenation experiments were carried out according to Prater and Haagen-smith (7).

## RESULTS

The data condensed in Tables I and II show that the samples used for the bioassays do contain the isolated double bond and are correctly designated as  $\gamma$ -carotene and poly-*cis*- $\gamma$ -carotene, respectively. The same is true of our samples of another poly-*cis* compound, *viz.*, polycyclopene,  $C_{40}H_{56}$ , originating from various sources.

In the case of some  $\gamma$ -carotene samples which were isolated from plant materials, of course, the possibility remains that some of the low

TABLE I

*Isopropylidene Group Estimations Using Some All-trans and Poly-cis Carotenoids*

Sample (and melting point)	Source	Weight of sample	Found acetone	Found isopropylidene groups/mole
		mg.	mg.	
All-trans Lycopene (173°)	Tomato	5.109	0.8757	1.6
Polycyclopene (112°)	<i>Pyracantha angustifolia</i>	10.70	1.8676	1.6
Polycyclopene (112°)	"Tangerine" tomato	9.20	1.6748	1.7
All-trans- $\gamma$ -Carotene (136°)	Pro- $\gamma$ -Carotene + I <sub>2</sub>	14.763	0.9289	0.6
All-trans- $\gamma$ -Carotene (129°)	<i>Pyracantha angustifolia</i>	8.987	0.8567	0.8
All-trans- $\gamma$ -Carotene (150°)	<i>Mimulus longiflorus</i> <sup>a</sup>	16.93	1.7085	0.9
All-trans- $\gamma$ -Carotene (150°)	<i>Mimulus longiflorus</i> <sup>a</sup>	22.12	2.2216	0.9
Pro- $\gamma$ -Carotene (135°)	<i>Pyracantha angustifolia</i>	15.038	1.2896	0.8
Pro- $\gamma$ -Carotene (135°)	<i>Pyracantha angustifolia</i>	15.025	1.5066	0.9
All-trans- $\beta$ -Carotene (180°)	Carrots	21.70	0.5399	0.2

<sup>a</sup> Reported earlier.

TABLE II  
Catalytic Hydrogenation of Poly-*cis* Carotenoids

Compound	Source	Sample weight	Catalyst weight	Hydrogen uptake (S.T.P.)	Found: double bonds mole
		<i>mg.</i>	<i>mg.</i>	<i>ml.</i>	
Polycopene	"Tangerine" tomato	6.257	6.46	3.38	13.0 <sup>a</sup>
Polycopene	"Tangerine" tomato	8.950	18.14	4.70	12.6 <sup>a</sup>
Polycopene	<i>Pyracantha angustifolia</i>	7.015	7.608	3.72	12.7
All- <i>trans</i> - $\gamma$ -Carotene	<i>Mimulus longiflorus</i>	5.943	3.04	2.94	11.9 <sup>a</sup>
All- <i>trans</i> - $\gamma$ -Carotene	<i>Mimulus longiflorus</i>	10.138	5.47	4.87	11.5 <sup>a</sup>
Pro- $\gamma$ -Carotene	<i>Pyracantha angustifolia</i>	7.013	5.304	3.34	11.4
Pro- $\gamma$ -Carotene	<i>Pyracantha angustifolia</i>	7.047	4.896	3.62	12.3

<sup>a</sup> Reported earlier.

melting points may have resulted from the presence of small amounts of an isomeric  $\gamma$ -carotene or of dihydro- $\gamma$ -carotene, the latter containing an isopropyl, instead of an isopropylidene, terminal group. This would be in accordance with the appearance of two, closely located " $\gamma$ -carotene" zones in some chromatograms (1). Furthermore, since phytofluene (4) and phytoflueneol (5) have been identified as highly hydrogenated carotenoids, it seems probable that there will also occur in nature some polyenes whose degree of saturation lies between that of phytofluene and the highly dehydrogenated carotenoids such as  $\gamma$ -carotene, lycopene, and their poly-*cis* forms.

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# The Determination of Nicotinic Acid

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## INTRODUCTION

During more than a decade, a number of papers have appeared describing apparently simple chemical procedures for the determination of nicotinic acid with cyanogen bromide (CNBr) and aromatic amines. The underlying reactions proceed in two steps: first, the intermediate production of a pyridinium derivative by reaction of the vitamin with CNBr, and second, the production of a colored glutaconic dialdehyde derivative by the subsequent reaction with the aromatic amine (1, 2). The various procedures have differed in many respects as follows: digestion of the sample with acids (3-7, 9-17, 19-21) or alkali (6, 8, 18, 19, 22-27); preparation of the extract by precipitation (4, 14, 15, 28, 29) or by removal of color and other interfering substances (3, 6, 9, 12, 14, 15, 20); reaction with CNBr at room temperature (3-7, 9, 17, 20, 28-34) or at elevated temperatures (8, 10-16, 18, 22-27). in neutral (3-7, 9, 17, 20, 21, 28-34) or slightly acid solution (8, 10-16, 18, 19, 22-27); development of color with a wide variety of aromatic amines (3, 23-26, 30, 33, 34) either in neutral (3-7, 9, 17, 20, 30-34) or acid solution (8, 10-16, 18, 19, 21-27); application of blank corrections by means of color blank (3-7, 14-17, 19, 34), CNBr blank (10, 23), amine blank (11, 18, 25), or by other means (21, 27). Although each author has presented data on the reliability of his procedure, chemical methods have not found as wide acceptance as the supposedly simpler and more specific, but slower, microbiological methods.

<sup>1</sup> This investigation was aided by the Clara A. Abbott Fund of Northwestern University. The authors express their appreciation to Professor Chester J. Farmer, Department of Biochemistry, for help generously given in the course of this study.

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The techniques still are complicated. The reactions in food extracts are more complex than in pure solution, and the results are subject to considerable variation. Many types of substances react with CNBr (35). Heated foods and acid digests contain decomposition products which yield color with aromatic amines. In each of the two steps, the rates of reaction and the stability of the resulting compounds are affected by the temperature, by the hydrogen-ion concentration, and by the concentration of reagents in the reaction mixture; small differences may alter the sensitivity and, hence, lead to variable results. In spite of these difficulties, the chemical and microbiological methods have yielded comparable results, with smaller variations by the chemical method, when carried out by equally experienced analysts.

In the present research, we have studied the reaction of CNBr with nicotinic acid and the production of color with various amines under somewhat different conditions than heretofore. Attention has been given to the interfering effect of CNBr- and amine-reactive substances in acid and calcium hydroxide extracts, and a method is suggested for correcting for the presence of these substances. On the basis of this work, a simplified procedure was evolved which should find application in the analysis of foods.

## METHOD

### *Reagents*

*Cyanogen Bromide (36), 0.5 M.* The preparation is carried out in a well-ventilated hood. Eighty g., approximately 27 ml., of cold bromine is weighed in a cold, tared 25-ml. cylinder and transferred to a glass-stoppered 1000-ml. volumetric flask containing approximately 500 ml. of cold water. The solution is cooled in a bath of ice water. A freshly prepared 10% solution of sodium cyanide<sup>3</sup> (NaCN) is slowly added from a glass-stoppered 250-ml. buret, with constant shaking during addition until the reaction mixture is almost decolorized. The walls of the flask are washed down with distilled water, after which the cyanide solution is added drop by drop until the reaction mixture is colorless. An excess of 10 large drops of cyanide solution is then added, and the volume is brought to the mark. The CNBr solution in the volumetric flask is kept in the refrigerator when not in use.

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<sup>3</sup> Merck reagent quality sodium cyanide of recent manufacture, and preferably from a newly opened small bottle, is recommended. After more than 2-3 years from the date of manufacture, the salt, even in unopened bottles, becomes progressively unsatisfactory for use in preparation of CNBr solutions. This is shown by decreasing yields of color when such CNBr solutions are used in analyses.

If the contents of an old NaCN bottle are used, the upper layer of salt to a depth of 2-3 cm. should be discarded. The solution should be filtered if not clear.

The reaction of bromine with the cyanide is quantitative and yields equimolecular quantities of CNBr and NaBr. Although the analytical results have shown no change with storage of solutions for as long as 1 yr., more frequent preparation is recommended.

*Buffered Cyanogen Bromide Reagent.* Cold CNBr solution, prepared as above, is poured into a 500-ml. glass-stoppered volumetric flask containing 25 g. potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ , C.P. or reagent quality). The volume is brought approximately to the mark with the CNBr solution after the salt has dissolved. The solution in the stoppered flask is stable for at least 2 months if kept in the refrigerator and protected from strong light. *The solution should be dispensed only from a buret. Pipets should never be used.*

*The Amine.* Metol (Photol, *p*-methylaminophenol sulfate) is preferred. Other amines, in the order of preference, are listed in Table I. The specified quantity of amine is dissolved in enough hydrochloric acid of the appropriate normality to bring the volume to 100 ml. The solution is prepared before use and is protected from strong

TABLE I

*Recommended Amines: Concentrations of Amine and Acid for Maximal Color Production. Effect of Structure of Amine*

Order of preference	Amine	Amine solution		Color density, Evelyn filter 400		
		Amine/100 ml. solution	HCl solution added	Stability within $\pm 1\%$ ; time after adding amine	Recommended time for readings	Average nicotinic acid equivalent of unit density
1	Metol <sup>a</sup>	g 8.0	N 0.5	min. 45-90	min. 60	$\mu\text{g}$ 19.6
4	<i>p</i> -Anisidine base	6.0	2.0	5-20	10	19.0
	<i>p</i> -Anisidine-HCl	8.0	1.5	5-20	10	19.0
	<i>o</i> -Aminophenol-HCl	6.0	1.0	15-30	20	24.6
	<i>m</i> -Aminophenol-HCl	6.0	1.0	8-25	10	20.4
5	<i>p</i> -Aminophenol-HCl	6.0	1.5	5-20	10	19.0
	Orthoform <sup>b</sup>	5.0	1.0	2-10	10	24.5
3	<i>o</i> -Toluidine-HCl	7.0	0.5	5-15	10	25.3
	<i>m</i> -Toluidine-HCl	7.0	1.5	5-25	10	19.6
	<i>p</i> -Toluidine-HCl	7.0	1.5	5-20	10	18.9
2	Aniline base	5.0	2.0	5-20	10	19.0
2	Aniline-HCl	6.5	1.5	5-20	10	19.0

<sup>a</sup> *p*-Methylaminophenol sulfate.

<sup>b</sup> Methyl-3-amino-4-hydroxybenzoate. Therefore, the compound is a substituted *o*-aminophenol.

light. Metol, *p*-aminophenol, and *p*-anisidine may be used throughout the day if kept cold; *p*-toluidine and aniline solutions may be used for several weeks if kept cold.

Metol is recrystallized as follows: 1500 ml. of approximately 0.1 *N*  $\text{H}_2\text{SO}_4$  are heated to boiling; 300 g. of Metol, previously mixed with 2 g.  $\text{NaHSO}_3$ , is added. The solution is heated to incipient boiling. If the solution is strongly colored, 25 g. of Norite is added. It is then filtered immediately in a large Buchner funnel which has been previously heated with boiling water. The filtrate is transferred to a large beaker containing 1 g.  $\text{NaHSO}_3$ ; 2 l. of 95% alcohol is added with stirring, and the solution is cooled in an ice bath. Isopropyl alcohol may be used. The crystallization is complete after cooling overnight in the refrigerator. The crystals on the Buchner funnel are washed with a spray of 95% alcohol from an atomizer, after which they are dried *in vacuo* over anhydrous calcium chloride. The preparation is stored in a brown glass-stoppered bottle protected from light. Yield: 85%.

*Calcium Hydroxide Suspension.* Approximately 500 ml. of distilled water is poured into a 3000-ml. beaker containing 250 g. calcium oxide, Merck reagent grade. The mass should be covered with water throughout the period of slaking. The mixture is stirred after the violent reaction has subsided; the beaker is covered, and the lime is allowed to slake further during the succeeding 3–4 hr. The lime is then suspended in water, and the small particles are completely disintegrated in the Waring blender. The creamy suspension is stored in a glass-stoppered bottle. The alkali content of the stock solution thus prepared is determined by titration with 1 *N*  $\text{HCl}$ , using phenolphthalein indicator.

For use in analysis, the stock suspension is diluted to an alkali content of 2 equiv. ml. The suspension is kept in a glass-stoppered bottle which is protected from dust and soot. The bottle is shaken well before each withdrawal.

*Zinc Sulfate Solution (28).* Eight hundred g. of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  is dissolved in hot water and diluted to a volume of 1 l. in a calibrated Erlenmeyer flask.

*Sulfuric Acid Solutions, 2, 5, and 10 N.*

*Hydrochloric Acid Solutions, 0.5 and 8.0 N.*

*Sodium Hydroxide Solutions, 1 N and 4 N.*

*Phenolphthalein Indicator.* A 1% solution in 70% ethyl alcohol.

*Caprylic Alcohol.*

*Potassium Dihydrogen Phosphate Solution.* A 5% solution is prepared at frequent intervals. The reagent is used in preparation of the amine and color blanks.

*Nicotinic Acid Standard.* Five hundred mg. of U.S.P. Reference Standard<sup>4</sup> which has been dried over  $\text{P}_2\text{O}_5$  is transferred to a 500-ml. volumetric flask; 5 ml. of 10 *N*  $\text{H}_2\text{SO}_4$  is added, and the volume is brought to the mark. This stock standard, which contains 1000  $\mu\text{g}$ . nicotinic acid/ml., is stable for at least 1 yr. if kept in the refrigerator and protected from light.

### Procedure

1. *Digestion of Sample.* Two g. of the air-dried, finely ground sample of cereal or plant tissues is suspended in 25 ml. 2 *N*  $\text{H}_2\text{SO}_4$  in a calibrated heavy-walled ignition tube of 25  $\times$  200 mm. dimensions. Larger samples are taken in the case of fresh

<sup>4</sup> Obtained from U.S.P. Reference Standards Committee, 4738 Kingsessing Ave., Philadelphia 43, Pennsylvania.

animal tissues, feces, or other materials which are not homogeneous and contain much water. In such instances, allowance is made for the water content: 5 ml. of 10 *N*  $\text{H}_2\text{SO}_4$  and enough water to bring the concentration to 2 *N* acidity are added.

If  $\text{Ca}(\text{OH})_2$  is used for digestion, the sample is suspended in 10 ml. of diluted lime suspension (equivalent to 20 ml. 1 *N* alkali) and the volume is brought to about 50 ml.

Blank tubes,<sup>5</sup> and tubes containing the sample and added quantities of nicotinic acid, are similarly prepared and carried through the entire procedure.

The suspension is now heated 90 min. in a boiling water bath. The contents are stirred frequently during the first few minutes to prevent lumping or massive coagulation; thereafter, the contents are mixed occasionally. The tube is then cooled in a bath of cold water, the volume is brought to exactly 50 ml., and the contents are mixed. The contents of the tube are clarified by centrifugation for 20 min. at 2000 r.p.m.

Acid digests of samples rich in fat should be extracted with chloroform before centrifugation. Much fatty substance not only hinders accurate adjustment of the volume, but also contributes substances which interfere in the later determination. After approximate adjustment of the volume to the 50-ml. mark, 5–10 ml. of chloroform is added and the contents are shaken vigorously for 5 min. After centrifugation, the volume of the aqueous phase may be calculated from two measurements: (a) the height of the supernatant aqueous column and (b) the distance between the 25- and 50-ml. marks.

Calcium hydroxide yields salts of sugars and starch which are quite insoluble in the cold. After adjustment of the volume to the mark, the contents are stirred frequently to break up the insoluble curd of calcium salts and to facilitate complete equilibrium. Clearer extracts are obtained if the tube is cooled in the cold bath before centrifugation.

2. *Neutralization, Clarification, and Partial Decolorization.* Twenty-five ml. or a suitable aliquot of the filtrate or supernatant solution is transferred to a second calibrated ignition tube. If lime is used for digestion, the aliquot is acidified by adding 0.5 ml. 8 *N*  $\text{HCl}$ . Two ml. of  $\text{ZnSO}_4$  solution, 2 drops of caprylic alcohol, and 2 drops of phenolphthalein indicator are added; 4 *N*  $\text{NaOH}$  is added slowly with constant stirring until a precipitate of  $\text{Zn}(\text{OH})_2$  begins to appear. If the solution becomes warm, it is cooled. One *N*  $\text{NaOH}$  is now added with constant stirring until a permanent pink color is obtained. Five *N*  $\text{H}_2\text{SO}_4$  is added drop by drop until the pink color disappears permanently, after which the volume is brought to 50 ml. The mixture is allowed to stand at least 10 min., during which time it is stirred occasionally to permit equilibrium. The contents may be filtered through 11-cm. Whatman No. 4 filter paper or centrifuged 20 min. at 2000 r.p.m. The latter is preferred. The solution may be stored in the refrigerator for 2 weeks without deterioration.

3. *Development of Color.* The analysis may require 4 sets of cork-stoppered Evelyn colorimeter tubes, A, B, C, and D, in order to correct for the color and amine- and  $\text{CNBr}$ -reactive materials as indicated below. Tube A contains all of the reagents; tubes B, C, and D serve as "amine," " $\text{CNBr}$ ," and "color" blanks, respectively.

<sup>5</sup> In routine analyses, a large volume of reagent blank may be prepared at one time and stored in a glass-stoppered bottle in the refrigerator.



Each set includes two tubes which contain 5 ml. of blank solution prepared from the reagents alone.

Five ml. of  $\text{Zn}(\text{OH})_2$  sample filtrate, containing from 1 to not more than 10  $\mu\text{g}$ . of nicotinic acid, is transferred to the stoppered tubes of sets A, B, C, and D. Each set with their respective reagent blanks are incubated about 10 min. in a water bath at  $50 \pm 2^\circ\text{C}$ . Tubes A and C then receive 2.0 ml. of buffered  $\text{CNBr}$  solution which is dispensed from a *buret*; tubes B and D receive 2.0 ml. of 5%  $\text{KH}_2\text{PO}_4$  solution. In order to assure accurate timing, the reagents are added at regular intervals, say, at 15- or 30-sec. intervals. The contents of each tube are mixed by rapid rotation, and the tube is returned immediately to the water bath. After incubating exactly 10 min. at  $50^\circ\text{C}$ ., each tube is placed in a water bath or large pan of water maintained at approximately  $25^\circ\text{C}$ .

After 10 min. of cooling, tubes A and B received 3.0 ml. of 8% Metol in 0.5 N  $\text{HCl}$ ; again, the reagents are added at regular intervals. Tubes C and D received 3.0 ml. of 0.5 N  $\text{HCl}$ . After mixing, each tube is returned to the water bath maintained at about  $25^\circ\text{C}$ . The tubes should be protected against strong light.

Photometric readings are made 60 min. after addition of Metol to tubes A and B. Readings may be made at 10-60 min. after addition of the acid to tubes C and D. Evelyn filter 400 is used.

If other amines are used, 3.0 ml. of solutions of the strength and acidity given in Table I is added, and readings are made at the times given in the table. Tubes C and D receive 3.0 ml. of  $\text{HCl}$  solutions of appropriate strength. Evelyn filter 400 is used.

TABLE II

*Removal of Interfering Substances and Color by Various Procedures*

Procedure	Color density					
	A All reagents	B Amine blank	C $\text{CNBr}$ blank	D Color blank	Net density $A - (B + C - D)$	Net amine reactive substances <sup>c</sup>
1. Neutralized with $\text{NaOH}$	0.403	0.411	0.092	0.101	+0.001	0.314
2. $\text{Zn}(\text{OH})_2$ precipitation	0.382	0.379	0.060	0.065	+0.008	0.316
3. Charcoal <sup>b</sup>	0.340	0.335	0.016	0.014	+0.003	0.320
4. Lloyd's reagent <sup>c</sup>	0.092	0.102	0.048	0.054	-0.004	0.052
5. $\text{Pb}(\text{OH})_2$ precipitation	0.332	0.330	0.011	0.014	+0.005	0.317
6. Lloyd's reagent; $\text{Pb}(\text{OH})_2$ precipitation <sup>d</sup>	0.038	0.038	0.002	0.002	0	0.036

<sup>a</sup> Authors' method.

<sup>b</sup> Melnick procedure with Darko charcoal and absolute alcohol.

<sup>c</sup> The supernatant alkaline solution after elution was neutralized and centrifugated.

<sup>d</sup> Perlzweig procedure.

<sup>e</sup> Net amine-reactive substances =  $A - \frac{C + D}{2}$ .

TABLE III

*Results Obtained by Digestion of Samples with 2 N H<sub>2</sub>SO<sub>4</sub>, Ca(OH)<sub>2</sub>  
or 4 N HCl Followed by Zn(OH)<sub>2</sub> Precipitation*

Duplicate samples were digested. Results represent averages of single determinations made on each Zn(OH)<sub>2</sub> filtrate. 8% Metol in 0.5 N HCl was used to develop color in tubes A and B.

Sample	Digestion with	Color density				Nicotinic acid		
		Colorimeter tube				Net color density	Recovery equivalent per unit density	In sample
		A All reagents	B Amine blank	C CNBr blank	D Color blank			
Oat flour	H <sub>2</sub> SO <sub>4</sub>	0.307	0.270	0.034	0.036	0.039	<i>μg.</i> 21.3	<i>μg. g</i> 8.3
	Ca(OH) <sub>2</sub>	0.053	0.015	0.011	0.010	0.037	20.5	7.6
Wheat flour	H <sub>2</sub> SO <sub>4</sub>	0.294	0.230	0.022	0.020	0.062	22.3	13.8
	Ca(OH) <sub>2</sub>	0.067	0.007	0.006	0.007	0.061	20.8	12.7
Soybean meal	H <sub>2</sub> SO <sub>4</sub>	0.735	0.638	0.099	0.106	0.104	19.2	20.0
	Ca(OH) <sub>2</sub>	0.144	0.042	0.039	0.030	0.093	20.5	19.1
Pea meal, yellow	H <sub>2</sub> SO <sub>4</sub>	0.658	0.523	0.055	0.055	0.135	22.1	29.9
	Ca(OH) <sub>2</sub>	0.152	0.022	0.018	0.015	0.127	21.5	27.3
Diet, composite	H <sub>2</sub> SO <sub>4</sub>	0.658	0.638	0.284	0.321	0.057		
	Ca(OH) <sub>2</sub>	0.138	0.066	0.065	0.055	0.062		
Pancreas	H <sub>2</sub> SO <sub>4</sub>	0.107	0.018	0.011	0.008	0.086	22.5	38.7
	Ca(OH) <sub>2</sub>	0.089	0.002	0.002	0.002	0.087	20.7	36.0
Feces 1 <sup>a</sup>	Ca(OH) <sub>2</sub>	0.284	0.055	0.080	0.045	0.194		
Feces 2	Ca(OH) <sub>2</sub>	0.216	0.063	0.065	0.045	0.133		
Feces 3	Ca(OH) <sub>2</sub>	0.277	0.063	0.087	0.051	0.178		
Blood 1 <sup>b</sup>	HCl	0.096	0.017	0.036	0.013	0.056		
Blood 2	HCl	0.102	0.016	0.033	0.017	0.070		
Blood 3	HCl	0.232	0.068	0.148	0.068	0.084		
Blood 4	HCl	0.223	0.060	0.145	0.075	0.093		

<sup>a</sup> Five-g. samples of feces were analyzed.

<sup>b</sup> Five ml. whole blood was digested 90 min. at 100°C. with 5 ml. 8 N HCl. The volume was diluted to 35 ml., and the digest was centrifugated. Twenty-five ml. of dark-brown supernatant solution was precipitated with Zn(OH)<sub>2</sub> and diluted to 50 ml.

*Blank Correction.* If the results of analysis of a given type of sample indicate the presence of amine- and CNBr-reactive substances, all blank corrections are applied. Some types of samples require only one blank. Examples are given in Table III. The color density, or  $L$ -value, is calculated from each reading obtained with tubes  $A$ ,  $B$ ,  $C$ , and  $D$ . Thus, if the instrument is set at a galvanometer reading of 100 units by means of the reagent blank,  $L = 2 - \log G$ , in which  $G$  is the galvanometer reading. Let  $A$ ,  $B$ ,  $C$ , and  $D$  represent the respective densities; then

$$\text{corrected } L = A - (B + C - D). \quad (1)$$

If the extract contains only amine-reactive substances besides the vitamin, a condition indicated by equal color densities in tubes  $C$  and  $D$ , then

$$\text{corrected } L = A - B. \quad (2)$$

On the other hand, if only CNBr-reactive substances are present, as indicated by equal color densities in tubes  $B$  and  $D$ , then

$$\text{corrected } L = A - C. \quad (3)$$

Finally, if all blanks have equal color density, correction may be applied by means of the color blank. This applies to many types of samples in which interfering substances are removed by the Perlzweig procedure, using Lloyd's reagent and  $\text{Pb}(\text{OH})_2$  precipitation. Thus,

$$\text{corrected } L = A - D. \quad (4)$$

## EXPERIMENTAL

### *Reaction with CNBr*

*Effect of Temperature.* As noted by Bandier (23, 24) and Harris and Raymond (25), the sensitivity of the method is increased by incubation of the nicotinic acid solution with CNBr at elevated temperatures. Thus, temperatures of 65–80° have been recommended. Under these conditions, the color density, obtained after cooling and subsequently adding the amine, rises to a maximum within 2–3 min.; it remains constant for a few minutes, and then declines. These conditions are not practical in routine work because only a few determinations can be made at one time. If colorimeter tubes are used, considerable condensation of moisture is noted in the upper part of the tube; this may affect the volume of solution unless the tube is tightly stoppered.

The maximal color density was not attained in 20 min. at 25°; it was noted after 10 min. incubation at 40°, after 6 min. at 50°, after 4 min. at 60°, and after 2 min. at 70°. A constant maximal color density was obtained during the following periods of incubation with CNBr: 10–25 min. at 40°, 6–15 min. at 50°; 2–6 min. at 60°; 2–4 min. at 70°. Thus, at the recommended 10-min. incubation period at 50°, CNBr

may be added to 39 tubes at intervals of 15 sec., or the reagent may be added to 19 tubes at intervals of 30 sec. Although accurate timing is advised, this is not so essential at 50° as at higher temperatures.

*Effect of Phosphate Buffer.* As noted by Bandier (23),  $\text{KH}_2\text{PO}_4$  buffer not only increases the specificity of the method in the presence of many other pyridine compounds, but also stabilizes the CNBr derivative over a longer period of incubation at 75–80° than without added buffer. All previous workers have added the acid phosphate separately. We have noted some differences in results which vary according to the time at which the phosphate is added to the reaction mixture. In the authors' procedure, a single solution containing the optimal quantities of CNBr and  $\text{KH}_2\text{PO}_4$  is added. This simplifies the technique, assures uniform conditions of hydrogen-ion concentration from the moment of addition of CNBr, and permits accurate timing of the period of digestion.

Plain CNBr solutions have relatively little buffering capacity. The color density depends not only upon the pH of the solution during digestion but also upon the buffering capacity of the solution. This buffering capacity affects the pH during development of color with the amine. The results, therefore, represent the over-all effect of the chosen conditions. The greatest color density, using Metol, was obtained with the authors' CNBr reagent. With plain CNBr only 40% as much color was obtained; with buffered CNBr containing 4 parts  $\text{KH}_2\text{PO}_4$  to 1 of  $\text{Na}_2\text{HPO}_4$ , only 60%; under both conditions, a considerable degree of stability of the CNBr derivative or derivatives was noted, as indicated by color development. At higher ratios of  $\text{Na}_2\text{HPO}_4$  in the reagent, the stability of the CNBr derivative or derivatives was apparently greatly reduced. Similar results were obtained when the color was developed with the more rapidly reacting primary amines.

#### *Reaction with the Amine*

*Effect of Acidity.* Teeri and Shimer (34) have noted a stabilizing effect of acid on the color produced after addition of the amine to the CNBr reaction mixture. The authors state that the acid must be added immediately after the amine; the color is reduced if the acid is added either before the amine or with the amine, as in our method. Apparently, only *m*-phenylenediamine gave satisfactory results. We have found that the final concentration of acid must be limited within a narrow range, and only a few amines can be used successfully if the reaction

of CNBr with nicotinic acid is carried out at room temperature at pH 6-7, as in the methods of Melnick and Field (6, 7), Teeri and Shimer, and others. On the other hand, many primary amines may be used successfully in acid solutions without affecting the color density materially if the reaction with CNBr is carried out at elevated temperatures in  $\text{KH}_2\text{PO}_4$  buffered solution. *Apparently, the reaction of CNBr with the vitamin is not a simple addition, because the products which are obtained at room temperature at pH 6-7 react differently with amines than the products which are obtained at elevated temperatures in  $\text{KH}_2\text{PO}_4$  buffer.*

Metol, the sulfate of a secondary amine, yields color of approximately the same stability and at approximately the same rate in aqueous and dilute acid solutions (23). However, the primary amines react quite differently, each in a characteristic manner. Aqueous solutions of the hydrochlorides or sulfates instantly yield maximal color which fades immediately. When the amines or their salts are dissolved in HCl solutions, the rate of color development is decreased progressively as the acidity is increased; simultaneously, the stability of the color is prolonged. It is thus possible to choose a suitable acidity at which the color density may be determined conveniently, say at 10 or more minutes after addition of the primary amine.

For example, on adding 0.5 *M* solutions of *p*-anisidine hydrochloride in 0.5, 1.0, 1.5, 2.0, and 2.5 *N* HCl, maximal color development was attained within 2, 4, 6, and 8 min. at 25°; the colors were stable, within  $\pm 0.5\%$  of the maximal color, during 2, 6, 14, 19, and 22 min.; the nicotinic acid equivalents per unit density were 18.8, 19.2, 19.2, 19.1, and 18.6  $\mu\text{g}$ . Identical results were obtained with 0.4 *M* solutions, but at lower acidities of HCl. Therefore, the results depend upon the concentration of the amine or its salt, as well as the acidity of the solution.

A large number of amines were thus studied with respect to the relation between the concentration of the amine and the acid. The recommended conditions for convenient reading of color density at 10 or more minutes after addition of several primary amines are given in Table I.

*Effect of Structure of Amine.* The aminophenols, the toluidines and the chloroanilines gave the following respective nicotinic acid equivalents per unit color density with Filter 400: ortho-, 24.6, 25.3, 28.3  $\mu\text{g}$ .; meta-, 20.4, 19.6, 20.3  $\mu\text{g}$ .; para-, 19.0, 18.9, 19.5  $\mu\text{g}$ . Orthoform, a substituted *o*-aminophenol, yielded a nicotinic acid equivalent of 24.5  $\mu\text{g}$ . The densities were lower (or the equivalents were greater) in the same

order when Filter 420 was used. Thus the *o*-compounds yielded less color than the *m*- or *p*-compounds; the *p*-compounds yielded about as much color as aniline, the unsubstituted compound (Table I). Of course, the agreement of results was due to some extent to the particular choice of concentrations of amine and HCl.

Metol yielded about 3% less color, but more stable color, than aniline or the *p*-substituted anilines. Thus substitution in the benzene ring materially affects the yield, but not the rate of production and stability of color; on the other hand, methylation of the amino group does not materially affect the yield of color, but greatly slows the rate of production and increases the stability of color.

### *The Blank Correction*

The greatest source of error in chemical assays lies in the blank correction. Examples of corrections applied with all blanks are given in Tables II and III. Thus, a diluted digest (Table II) prepared from Bureau of Standards cane sugar yielded a net density of +0.001, in spite of the high amine blank, which contained more color than tube A. Such a result is commonly obtained with pure sugar digests; however, the CNBr blank also is usually lower than the color blank, an observation which indicates that CNBr may react with colored substances to yield substances with less color. The close agreement of net color densities in Table III, in spite of extreme differences in amine blanks in extracts prepared by acid and alkaline digestions, should be noted. The consistency of results in both tables demonstrates that the method is valid, although not perfect. In the case of the first 4 samples in Table III, results of similar consistency were obtained by corrections applied with the amine blank alone according to Eq. (2) under *Methods* above.

### *Removal of Color and Reactive Substances*

The effect of several procedures is shown by an experiment with a cane sugar digest (Table II).  $\text{Zn}(\text{OH})_2$  precipitation removed less color (column 5) than charcoal or  $\text{Pb}(\text{OH})_2$  precipitation; neither removed amine-reactive substances (column 7). *Therefore, the removal of colored substances was independent of the removal of amine-reactive substances.* The use of Lloyd's reagent as recommended by Perlzweig removed about 50% of the color and about 85% of the amine-reactive substances. The combined use of Lloyd's reagent and  $\text{Pb}(\text{OH})_2$  precipita-

tion, as in the Perlzweig procedure (14), removed 98% of the color and about 90% of amine-reactive substances.

In our hands, the use of charcoal as specified by Melnick and Field, has led to large losses of nicotinic acid from pure solutions;  $\text{Pb}(\text{OH})_2$  precipitation, alone or in combination with Lloyd's reagent, has occasionally given small and variable losses. No loss has been observed with  $\text{Zn}(\text{OH})_2$  precipitation. As shown by Somogyi (37),  $\text{Zn}(\text{OH})_2$  removes many types of substances. The gelatinous precipitate is an excellent clarifying agent.

### *Digestion of Samples*

At equivalent concentrations, digestion of samples with  $\text{HCl}$  yields more color and amine-reactive substances than with  $\text{H}_2\text{SO}_4$ . Digestion with 2 *N*  $\text{H}_2\text{SO}_4$  for 90 min. at 100°C. effects complete conversion of nicotinamide and coenzymes (but not nicotinuric acid) into nicotinic acid and, therefore, may be used for most biological materials.  $\text{Ca}(\text{OH})_2$  does not convert nicotinamide into nicotinic acid as rapidly as 2 *N*  $\text{H}_2\text{SO}_4$ . The apparent recovery of nicotinic acid from  $\text{Ca}(\text{OH})_2$  digests of samples varies from 91–6% with an average of 95%, and is greater and more uniform than from  $\text{H}_2\text{SO}_4$  digests (column 8, Table III). The differences in recovery from acid digests may be due to interferences in color development or to greater errors introduced by the relatively higher blanks in the acid digests.

$\text{Ca}(\text{OH})_2$  yields practically no amine-reactive materials. Thus, the sample of oat flour (Table III) yielded 0.015–0.010, or 0.005, color density due to amine reaction; with  $\text{H}_2\text{SO}_4$  it yielded 0.270–0.036, or 0.234, color density. The differences between acid and alkaline extracts usually are of this order with plant materials. The net color densities obtained by acid and alkali digestions show satisfactory agreement; in the case of the 5 examples cited in Table III, still better agreement was obtained by correction with the amine blank alone. However, acid digestion generally yields a higher calculated nicotinic acid content of the sample than  $\text{Ca}(\text{OH})_2$  digestion when the respective factors for nicotinic acid recovery are used in the calculations. The  $\text{Ca}(\text{OH})_2$  digestion may be preferable for the analysis of starchy plant materials and certain materials of animal origin because the extracts contain fewer interfering substances, and therefore require fewer blanks and smaller corrections by blanks; also, because the corrected color densities are approximately equal to those obtained by acid digestion.

## SUMMARY AND CONCLUSIONS

1. A procedure has been described for the preparation of sample extracts and the subsequent determination of nicotinic acid by the reaction with  $\text{KH}_2\text{PO}_4$ -buffered  $\text{CNBr}$  at  $50^\circ$  and the development of color with Metol or other amines in acid solution.

2. Extracts of foods may contain three categories of substances which affect the accuracy of the results: colored substances, those which yield color with the amine, and those which react with  $\text{CNBr}$ . Directions are given for evaluation of the relative quantity of each, and the correction of the results by color, amine, and  $\text{CNBr}$  blanks, respectively. The yield of the three classes of interfering substances from various types of samples is affected by the method of digestion, whether in acid or alkaline medium. Methods for removal of interfering substances are discussed.

3. Directions are given for purification of Metol and for preparation of stable buffered  $\text{CNBr}$  and other reagents. The presence of  $\text{KH}_2\text{PO}_4$  during reaction of  $\text{CNBr}$  with nicotinic acid at  $50^\circ$  enhances the stability of the  $\text{CNBr}$  derivative and increases the sensitivity and the specificity of the method for nicotinic acid.

4. The effect of structure of primary aromatic amines on the rate of development and stability of color was studied. Each amine reacts in a characteristic manner and requires a definite concentration and acidity for producing maximal stable color. The rate of development and the stability of color with Metol, a secondary amine, is not affected materially by the acidity of the medium. A list of amines is given, with the order of preference specified, for use in development of color after reaction of nicotinic acid with  $\text{CNBr}$  at  $50^\circ$ .

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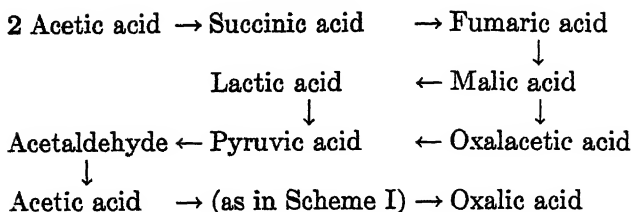
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## Scheme II



It was reported recently (6) that  $\alpha$ -ketoglutaric acid and oxalacetic acid may undergo a dismutation in the presence of enzyme preparations from kidney cortex whereby the former is oxidized to succinate by the latter. In view of this postulation, and since our wood-destroying molds are known to possess a strong dehydrogenating enzyme system (5), Dr. Nord suggested a study of the function, and an attempt to decide the course of conversion, of  $\alpha$ -ketoglutaric acid during the progress of the carbohydrate metabolism.

## EXPERIMENTAL

$\alpha$ -Ketoglutaric acid was prepared according to standard procedures (7,8), and was quantitatively determined by applying the 2,4-dinitrophenylhydrazine method (9), using an Evelyn colorimeter with a 440  $m\mu$  filter.

The organisms studied in this investigation were the wood-destroying species *Trametes cinnabarina* (Tracina) and *Lentinus lepideus* (Lelep), both obtained from the New York Botanical Garden through the courtesy of Dr. Wm. J. Robbins. Lelep was subcultured on 2% glucose agar plates, while with Tracina 2% malt agar was used. Two ml. of a spore mycelial suspension in sterile distilled water were applied as inoculum.

A medium of the following composition was employed:

$\alpha$ -Ketoglutaric acid	10.0 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g.
$\text{KH}_2\text{PO}_4$	1.5 g.
Neopeptone <sup>a</sup>	1.0 g.
Urea <sup>b</sup>	0.3 g.
Thiamine hydrochloride	2.0 mg.
Tap water to	1.0 l.

<sup>a</sup> For Lelep; <sup>b</sup> for Tracina.

The media were sterilized by autoclaving at 15 lb. pressure for 20 min. Substances likely to be affected by autoclaving (such as  $\alpha$ -ketoglutaric acid and urea) were sterilized separately by Seitz filtration and added aseptically. The media were brought to a pH of 4.5 with NaOH.

Periodic analyses were made after inoculation, and were carried out in duplicate. Mycelial weights were determined by filtration through tared alundum crucibles, drying at 60°C. overnight and then reweighing. Ethyl alcohol was determined as usual (10), possible interfering aldehydic substances being previously removed (11). The pH values of the media were determined with a Cambridge electron-ray pH meter. Oxalic acid was determined permanganimetrically (12). The molds were inoculated into the above medium and incubated in the dark at 28°C.

The following results were obtained:

TABLE I  
*Dissemination of  $\alpha$ -Ketoglutaric Acid*

Organism	Days	$\alpha$ -KG <sup>a</sup>	Oxalic acid <sup>b</sup>	EtOH <sup>c</sup>	Mycelium <sup>d</sup>	pH
Lelep	0	1000	—	—	—	4.5
	32	800	0.0	nil	62.0	4.7
	60	750	11.6	nil	65.6	4.9
Tracina	0	1000	—	—	—	4.5
	28	910	0.0	nil	51.6	4.8
	56	410	106.0	nil	58.6	6.6

<sup>a</sup>  $\alpha$ -Ketoglutaric acid in mg.-%.

<sup>b</sup> Oxalic acid in mg.-%.

<sup>c</sup> Ethanol.

<sup>d</sup> Mycelium in mg. 100 ml.

The yields of oxalic acid produced are recorded in Table II. Volatile acids were not detectable in the culture filtrates.

TABLE II  
*Yields of Oxalic Acid Formed*

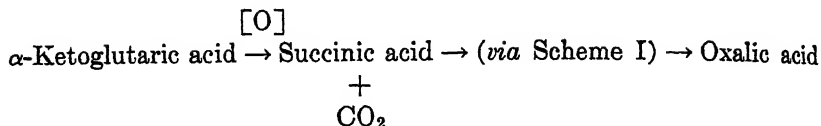
Organism	mMoles of $\alpha$ -KG consumed <sup>a</sup>	mMoles of oxalic acid formed	Days
Lelep	approximately 1	0.13	60
Tracina	3.4	1.18	56

<sup>a</sup>  $\alpha$ -Ketoglutaric acid.

## DISCUSSION

From the above results, several possible mechanisms for the breakdown of  $\alpha$ -ketoglutaric acid are conceivable:

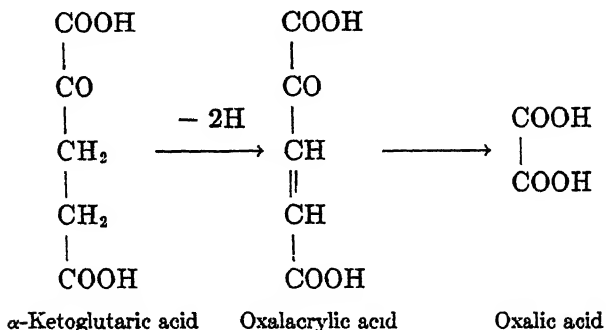
## Scheme III



## Scheme IV



## Scheme V



Scheme III shows an oxidative decarboxylation of  $\alpha$ -ketoglutaric acid to succinic acid, from which oxalic acid could be produced *via* previously established pathways, as in Scheme I. Scheme IV shows a direct hydroclastic split of  $\alpha$ -ketoglutaric acid to oxalic and propionic acids, while Scheme V indicates an initial dehydrogenation of  $\alpha$ -ketoglutaric acid.

Scheme IV is militated against by the fact that the propionic acid which would be produced is considered as toxic to certain fungi (13). Furthermore, the presence of strong dehydrogenating systems in these molds (5) makes it quite possible that some dehydrogenation takes place.

In an attempt to distinguish between Schemes III and V above, it was considered that the dehydrogenation indicator resazurin might be of value. Accordingly, experiments employing it in the above  $\alpha$ -ketoglutaric acid medium were performed.

Resazurin was purified by treatment with  $\text{Na}_2\text{O}_2$ , as in the synthesis of this substance previously reported (5), and it was incorporated in the medium to give a concentration of  $1.5 \times 10^{-5} M$ .

The absorption spectra of the purified resazurin and resorufin (5) were determined at pH's 4.0, 6.0, and 8.0; the maxima and minima recorded are shown in Table III.

TABLE III  
*Absorption Spectra of Resazurin and Resorufin*

	pH			pH		
	4.0	6.0	8.0	4.0	6.0	8.0
	Maxima			Minima		
Resazurin	260	280	285	300	272	272
	360	378	378	382	402	—
	520	590	590			
Resorufin	475	570	560	310	340	350

These data served as a basis for the evaluation of the progress of the observed dehydrogenations.

The media were adjusted to pH 5.5 with NaOH.

The following media were prepared with the carbon sources as indicated:

- A. 0.5%  $\alpha$ -ketoglutaric acid + resazurin.
- B. 0.5% succinic acid + resazurin.
- C. 0.5% succinic acid + resazurin + malonic acid 0.25, 0.5, and 1.0%.
- D. 0.5%  $\alpha$ -ketoglutaric acid + resazurin + malonic acid 0.1, 0.25, 0.5, and 1.0%.

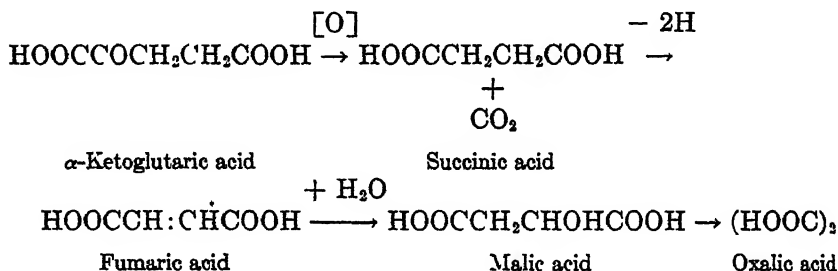
If Scheme III were to be the one availing, then Series A and B should show discoloration of the indicator, while Series C should show progressively decreasing amounts of dehydrogenation due to the increasing quantities of succinic dehydrogenase inhibitor. Series D should show the same results. On the other hand, if Scheme V should be prevailing, Series A and D should show equally intense dehydrogenation, regardless of the presence of inhibitor. However, if only Scheme

IV is involved, only Series *B* and *C* should show discoloration of the medium.

The first of these possibilities was found to be the case with *Lelep*. Hence we may say that with this organism, Scheme III seems to be favored. On the other hand, the results with *Tracina* were anomalous, since all series, whether with  $\alpha$ -ketoglutaric acid or with succinic acid, regardless of the presence or absence of inhibitors, showed equally intense dehydrogenation. This result may have been due to some endogenous respiration, independent of the substrate added.

In the case of *Lelep* at least, the formation of oxalic acid from  $\alpha$ -ketoglutaric can be visualized as proceeding *via* a scheme as follows:

#### Scheme VI



As may be seen, the first step of this phase sequence parallels the action of the enzyme from heart muscle which was described by Ochoa (14). The demonstrated intervention of malonic acid in the dehydrogenation further supports the concept of the formation of oxalic acid *via* a direct decarboxylation and subsequent dehydrogenation rather than by hydroclastic split of oxalosuccinic acid to oxalic and succinic acids, as was proposed recently (15).

With *Tracina*, however, although this mechanism is not excluded, a direct dehydrogenation is equally possible on the basis of the evidence presented.

#### SUMMARY

The formation of oxalic acid from  $\alpha$ -ketoglutaric acid by wood-destroying molds has been experimentally evidenced. A pathway of dehydrogenation reactions is indicated, *via* succinic acid and other four carbon acids involved in the genesis of oxalic acid from  $\alpha$ -ketoglutaric acid.

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## On The Mechanism of Enzyme Action. XLII. Isolation and Some Properties of the Lipase from *Fusarium lini* Bolley

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### INTRODUCTION

Enzyme studies with fusaria have been conducted quite extensively for many years. About fifteen years ago, it was observed (33) that *Fusarium lini* Bolley (FLB) grown in surface culture possesses a high fat content. Various studies regarding carbohydrate metabolism, intermediary metabolism, alcoholic fermentation, dehydrogenations, fat formation and pigment production, have been reviewed elsewhere (15, 36, 37). With these investigations completed, the foundation had been laid for studies involving the conversion of carbohydrate to fat. Thus, the composition of fusaria fats was established for the first time (12, 31), and at the same time, a knowledge of the structure of some natural pigments that occur in these molds was gained (30, 49). It was also observed that these natural products can influence enzymatic actions in these fungi (30, 43), especially the powerful dehydrogenations that can be brought about by fusaria (32).

These natural pigments were then shown to be interrelated in the mechanism of carbohydrate  $\rightarrow$  fat conversion both quantitatively (35, 49) and qualitatively (9), and their actions were compared with several vitamins, which can act in enzymatic dehydrogenations (34). Subsequently, an investigation of the enzyme involved in the formation

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and degradation of neutral fats led to the finding that FLB possesses a true lipase (13).

It was thus our purpose to attempt an isolation of the enzyme from a source hitherto unrecorded; *i.e.*, from FLB, in order to contribute to our understanding of the carbohydrate  $\rightarrow$  fat conversion. At the same time, a possible answer has been sought as to why it has thus far been impossible to obtain the enzyme in crystalline form.

## METHODS

### (a) Microbiological Conditions

As heretofore, our strain, No. 5140, was employed in these investigations and stock cultures of the organism were maintained on the customary nutrient medium (41). The FLB was always grown on a Raulin-Thom medium containing 2.5% glucose (12). The dry mats, obtained as previously described (12), were ground to 40-mesh and stored in a vacuum desiccator over conc. c.p.  $H_2SO_4$  at  $-14^\circ C$ . for further drying. The material so obtained showed no loss in lipolytic activity even after 24 months of storage.

### (b) Analytical Methods

*Total Solids (T.S.)*. A small beaker containing approximately 1 g. of sea sand is dried to constant weight in an oven at  $100^\circ C$ . To this is then added a definite amount (*e.g.*, 3 ml.) of the enzyme suspension or extract, and the whole is placed in an oven at  $100^\circ C$ ., evaporated, and dried to constant weight.

*Kjeldahl Nitrogen (K.N.)*. Kjeldahl nitrogen was determined by a modification of the micro-method of Ma (22). The analysis was usually performed volumetrically; *i.e.*, on 1 ml. of enzyme material containing no more than 10 mg. T.S./ml.

### (c) Techniques

*Lyophilization*. A simple method (14) was employed for lyophilization or freeze-drying. A portion of the material to be so treated was suspended in distilled water in pyrex flasks and rapidly frozen in a dry-ice-acetone bath. The flasks were then placed in a vacuum desiccator (over conc. c.p.  $H_2SO_4$ ) which was then evacuated to about 2 mm. pressure. The desiccator was then stored at  $-14^\circ C$ . until the desired material was dry.

*Centrifugations* were carried out at 2000 r.p.m.

## EXPERIMENTAL

### I. Quantitative Method for Determining Lipase Activity

During the course of the isolation work, it was decided to use a modification of the method of Bullock (6). Advantages in the use of triacetin as the test substrate have been pointed out (10, 48). However, the tendency of the triglyceride to undergo auto-

hydrolysis in the presence of water and hydroxyl ions somewhat restricts its use (47). The method employed is as follows:

*Reagents:*

1. Triacetin (5% by vol. in distilled water, brought to pH 7.0 with 0.1 *N* NaOH).
2. McIlvaine buffer, (24) pH 7.0.
3. 1% alcoholic phenolphthalein.
4. 0.01 *N* aqueous NaOH.

*Procedure:*

Ten ml. of the triacetin solution is placed in a 125-ml. Erlenmeyer flask and to it is added 0.5 ml. of McIlvaine buffer (pH 7.0). After shaking gently, 2 ml. of enzyme suspension or extract is added and the contents again shaken. The flask is stoppered and incubated at 37°C. for 3 hr. with constant shaking. At the end of this time, 10 ml. of distilled water are added, followed by 0.2 ml. of 1% alcoholic phenolphthalein and the whole is titrated with 0.01 *N* NaOH.

Each analysis is conducted in duplicate with a duplicate control. The controls are prepared by bringing the enzyme suspension or extract to a boil and cooling. This treatment is sufficient to inactivate the enzyme completely.

## II. pH Optimum of FLB Lipase

The pH curve and the temperature curve were determined using both a water extract and a glycerol extract of dry, undefatted FLB. The McIlvaine buffers, which afford a pH range of 2-8, were used in this study. The extracts were prepared as follows:

*Aqueous Extract.* Ten g. of dry, undefatted FLB are placed in a Knapp-Monarch (K. M.) blender with 200 ml. distilled water (at high speed) for 3 min. at room temperature. The contents are then centrifuged for 20 min., and the supernatant decanted. This cloudy liquid is termed the "aqueous extract."

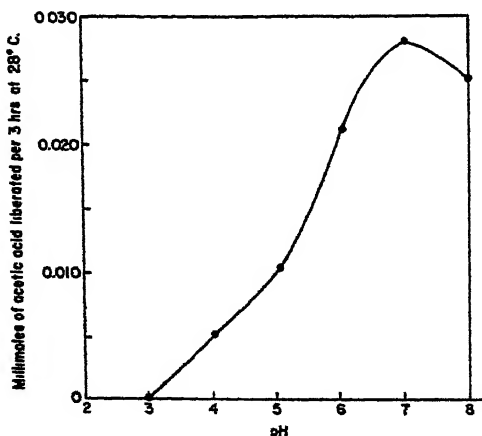


FIG. 1. pH Optimum of a Water-extract of FLB Lipase.

*Glycerol Extract.* Ten g. of dry, undefatted FLB are suspended in 100 ml. of 80% glycerol with occasional stirring (at 28°C.) for 8 hr. At the end of this time the mixture is centrifuged for 20 min. and the supernatant is decanted. This viscous liquid is again centrifuged and decanted. This material is termed the "glycerol extract" and is similar to the one prepared by Willstatter (50).

Using these two preparations comparable results were obtained in the determination of the pH curve at a constant temperature of 28°C. However, since the values obtained with the water extract were higher than those of the glycerol extract, it was decided to present these data alone (Fig. 1).

It can, therefore, be concluded that the FLB lipase is most active at pH 7.0 and between pH 6.5 and 8.0. In this respect, it resembles cottonseed lipase (39), and is unlike castor bean lipase (20) and the lipase of *Penicillium oxalicum* (19).

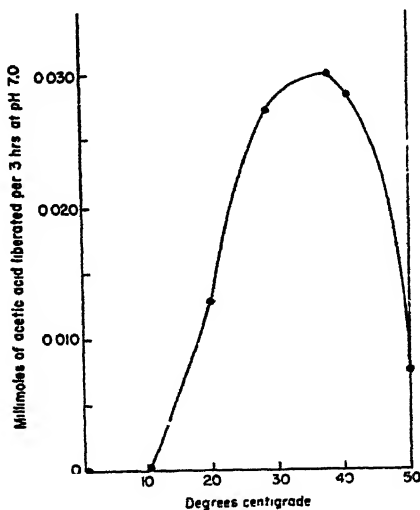


Fig. 2. Temperature Optimum of a Water-extract of FLB Lipase.

### III. Temperature Optimum of FLB Lipase

In order to find the optimum temperature for lipolytic activity, the aqueous and glycerol extracts referred to above were employed. Again, with both preparations, similar results were obtained employing a constant pH of 7.0. The results of this study, obtained with the aqueous extracts, are presented in Fig. 2. Thus FLB lipase is most active at 37°C.

### IV. Qualitative Method for the Detection of Lipase

Esterases are capable of rapidly hydrolyzing the colorless acyl esters of *p*-nitrophenol to produce the yellow-colored substance, *p*-nitrophenol. Were the FLB lipase capable of effecting such a hydrolysis, this color change could be used for a rapid

qualitative test. The acetate was prepared according to the method described (18) and the use of this ester with pancreatic lipase and FLB lipase was found to produce an instantaneous and sensitive test for the detection of lipase.

### V. Definitions

The definitions of Lipase Unit, Lipase Value, Concentration, and Purification as used in this paper are as follows:

*Lipase Unit (L.U.).* That amount of enzyme material which liberates 0.01 millimoles of acetic acid (equivalent to 1 ml. of 0.01 *N* NaOH) when allowed to act on 10 ml. of triacetin (5% by volume in distilled water) and 0.5 ml. McIlvaine buffer at pH 7.0, 37°C. for 3 hr., the final volume being 12.5 ml.

*Lipase Value (L.V.).* The number of lipase units present in 1 g. of the dry enzyme preparation.

*Concentration.* L.U./mg. T.S.

*Purification.* L.U./mg. K.N.

### VI. FLB Lipase—General

When working with a fatty cell, it is important, after a preliminary grinding, to defat the meal in order to make the enzyme accessible to various extracting solvents. The effect of various fat-solvents when applied to the defatting of the FLB mycelium using a 24-hr. extraction period and a large Soxhlet apparatus was therefore tested.

The FLB lipase [as other lipases, (47)] is very stable in the dry state. Thus, the mycelium, as a dry powder can be kept at 100°C. for 3 hr. with a loss of only 50% of its activity. On the other hand, the lipase is extremely unstable in the presence of water, as has been pointed out in regard to other lipases (5, 7, 19, 21, 41), this phenomenon being a function of temperature (23).

The defatting operations had to be carried out therefore, under very stringent and anhydrous conditions. Thus it was found that defatting with redistilled  $\text{CHCl}_3$ , 13% loss in lipase activity occurred; with anhydrous low-boiling petroleum ether, 4% loss, and with anhydrous ethyl ether, no loss in lipolytic activity was detected. Slight traces of moisture increased lipase losses. Anhydrous ethyl ether has been used in all succeeding studies in order to defat the dry and finely ground FLB mycelium. This method, of course, affords a more complete fat extraction than operations which are performed in the cold (3).

In order to find the most suitable extracting agent for the lipase, various ones were tried using dry, ethyl-ether defatted FLB. This is shown in Table I.

In each case 2 g. of the defatted FLB were suspended in 100 ml. of the extractant in a K. M. blender (high speed) at room temperature for 1 min. The mixture was then immediately centrifuged for 25 min. (see Table Ia), or incubated for 3 hr. at 37°C. with constant shaking and then centrifuged for 25 min. (This procedure was followed for samples shown in Table Ib.) After centrifugation, 80 ml. of each supernatant was brought to pH 7.0 using 1 *N* NaOH or 1 *N* acetic acid ( $\text{HOAc}$ ) and diluted to 100 ml. before testing.

The 50% egg white was prepared by diluting egg white with an equal volume of distilled water. The literature indicates that the instability of lipases in aqueous solu-

tions is probably due to the proteolytic activity of trypsin. Consequently, since egg white has been reported to contain antitrypsin (1), an attempt was made to extract and stabilize the enzyme simultaneously.

It should be noted that the calculations are based on the fact that 1 g. of the dry powder used in these studies contained 55.5 L.U. In Table I, distilled water gave 0.72 L.U. ml., therefore the percentage lipase extracted is calculated as follows:

$$0.72 \times 10/8 \times 100/111 = 81\%$$

Table I shows that distilled water is the best extracting agent. In fact, in order to see whether more lipase could be extracted with distilled water, 2 g. of the FLB powder were suspended in 100 ml. of distilled water for 5 min. at room temperature in a K. M. blender and centrifuged 25 min. The supernatant was treated as before and it was found that 95–100% of the lipase could be extracted under these conditions.

TABLE I  
*Efficiency of Various Agents in Extracting FLB Lipase*

Extractant	(a) Without Incubation		(b) After 3 Hr. Incubation at 37°C.	
	L.U./ml.	% Lipase extracted	L.U. ml.	% Lipase extracted
50% Egg white	0.00	0	0.00	0
80% Glycerol	0.20	22	0.40	45
10% NaCl	0.40	45	0.42	48
30% Dextrose	0.56	63	0.54	60
10% NaOAc	0.57	65	0.64	72
0.025 N NH <sub>4</sub> OH	0.64	72	0.68	76
Distilled water	0.72	81	0.44	50

Even though water is the best extractant, Table I(b) gives evidence to the fact that the lipase is stable in all the other cases in the presences of salts, etc., but loses about 50% its activity at 37°C. after 3 hr. in aqueous solutions. It will be seen also that (1) egg white allows no extraction of the lipase; this is probably due to the high protein concentration already present in the egg white, and (2) glycerol alone shows an increase in lipase activity after incubation at 37°C. This is probably due to the high viscosity of the glycerol which decreases its penetration into the cell, thereby making it a slower acting extracting agent.

A consideration of all known facts (26, 41, 45, 47, 51) made it advisable to use pure distilled water as the extractant and to adopt a policy of keeping the lipase in solution the minimum amount of time in all subsequent operations. Since the primary purpose was to obtain a highly concentrated and purified lipase preparation, no attempt has been made to calculate lipase losses at any stage of its isolation.

In order to have a reference standard, the lipase content of dry, ethyl-ether defatted FLB was determined. In this case, instead of using a suspension of the FLB in water or an aqueous extract of this material, the determination was performed on the

dry mat itself. Two samples and two controls were run simultaneously. One hundred mg. of the powder was weighed directly into 125-ml. Erlenmeyer flasks. After adding 2 ml. of distilled water to each flask, the contents were stirred gently. To the two samples were added 10 ml. triacetin (5% by volume, pH 7.0) and 0.5 ml. McIlvaine buffer (pH 7.0). The triacetin and buffer of the controls were placed in separate flasks and were added to the powder just prior to titration. All flasks were incubated at 37°C. for 3 hr. and then titrated as usual. Kjeldahl nitrogen determinations were run on separate samples of the same powder. The results are listed below:

Material: Dry, ethyl-ether defatted FLB.

Total solids: 100 mg.

Lipase units: 2.63.

Kjeldahl nitrogen: 7.4 mg.

Lipase value: 26.3.

L.U./mg.: 0.4.

### VII. Concentration and Purification of FLB Lipase

Lipase has never been obtained from either plant or animal sources in crystalline form. Attempts to confirm a claim (2) of having obtained crystals 2 mm. long from a pancreas lipase extract have been unsuccessful (5). However, it has recently been reported (28) that liver esterase has been obtained in crystalline form, though an earlier attempt to isolate pancreatic esterase failed (42).

*Isolation of FLB Lipase.* From the maze of techniques applied to the FLB lipase, there have emerged two procedures for its concentration and purification. These methods have been presented in Schemes I and II. The activities of the various fractions obtained during the course of these studies are presented in Table II.

TABLE II

*Activity of FLB Lipase at Various Stages of Concentration and Purification*

Scheme no.	Stage	T.S. mg./ml.	K.N. mg./ml.	L.U./ml.	L.V.	L.U./mg. K.N.
I	I (R.S.)*	100.0	7.40	2.63	26	0.4
	II (Supernatant)	21.0	2.30	1.74	83	0.8
	III (Residue)	14.0	2.40	0.09	7	0.04
	III (Supernatant)	10.0	0.80	2.10	210	2.6
	V (Residue)	9.7	0.78	1.90	200	2.4
II	II (Residue)	22.3	2.45	1.27	57	0.5
	II (Supernatant, after step 2)	8.9	0.55	2.50	281	4.6
	III (Residue)	6.3	0.36	2.30	365	6.4

\* R.S. = Reference standard. This determination was carried out directly on the solid.

# Scheme I

*Stage I.* Thirty g. of the dry, ethyl-ether defatted FLB (L.V. 26.3, 0.4 L.U. mg. K.N.) are placed in a K. M. blender for 5 min. (low speed) with 400 ml. of distilled water at room temperature and centrifuged for 25 min.

*Stage II.* The residue is discarded even though the lipase is not completely extracted, since it was found that further treatment with water complicates matters by yielding more proteins and little lipase.

## SCHEME I

### Isolation of FLB Lipase

Stage I

Dry, Et<sub>2</sub>O-Defatted FLB Mycelia  
(L.V. 26.3, 0.4 L.U./mg. K.N.)

1. Suspend in H<sub>2</sub>O, at room temp. for 5 min. in a blender.
2. Centrifuge 25 min.

Stage II

Residue (discard)

Supernatant

1. Clarify by cooling to 0°C., adding cold 1 N HOAc to pH 3.0.
2. Centrifuge 25 min.

Stage III

Residue (discard)

Supernatant

1. Cool to 0°C. and add slowly with stirring to 3 vols. 95% alcohol at -50°C.
2. Centrifuge 20 min.

Stage IV

Residue

Supernatant (discard)

1. Desiccate with acetone at -10°C.
2. Centrifuge 15 min.

Stage V

Residue

Supernatant (discard)

1. Dry *in vacuo*, over conc. c.p. H<sub>2</sub>SO<sub>4</sub> at -14°C.
2. L.V. 200, 2.4 L.U./mg. K.N.  
(This material is known as the Active Alcohol Precipitate.)



The very cloudy supernatant or initial water extract has a pH of 6.6 to 6.8. As in the case of wheat germ lipase (47), this coincides with the enzyme's pH of optimum stability and with the pH of its natural habitat. The enzyme is not destroyed between pH 5 and 11, although activity falls rapidly at the upper end of this scale. However, the enzyme is extremely unstable in water at any pH. It has been found that such aqueous extracts can be immediately frozen with dry-ice and acetone and stored indefinitely at  $-14^{\circ}\text{C}$ .

The use of saturated or solid  $(\text{NH}_4)_2\text{SO}_4$  at various concentrations and pH values, at  $37^{\circ}\text{C}$ . or low temperature (4), did not allow fractionation of the turbid suspension. However, it was found that the cloudy supernatant (250 ml.) could very easily be clarified without much loss in activity, by cooling to  $0^{\circ}\text{C}$ . and adding to it slowly 1 N HOAc ( $0^{\circ}\text{C}$ .) with mechanical stirring until a pH of 5.0 is obtained (about 5 ml.). Following this, the whole is centrifuged 25 min.

*Stage III.* The residue containing little activity (Table II) is discarded and the supernatant is again cooled to  $0^{\circ}\text{C}$ . It was found that even by lowering the pH of the supernatant to 4.0, it could not be further clarified and only resulted in large lipase losses. The residue was high in K.N. which means that by acid precipitation several protein impurities have been removed.

The supernatant (kept at  $0^{\circ}\text{C}$ .) is then added slowly with stirring to 3 volumes of 95% ethyl alcohol which have been previously cooled to  $-50^{\circ}\text{C}$ . in a dry-ice-acetone bath. During the procedure the temperature of the mixture is not allowed to rise above  $-10^{\circ}\text{C}$ . The mixture is immediately centrifuged for 20 min. At the end of this time, the temperature of the mixture is approximately  $5^{\circ}\text{C}$ .

*Stage IV.* The yellow, fluorescent, riboflavin-containing (34) supernatant (from which a very small amount of active material can be recovered) is decanted and discarded.

The residue is then desiccated with acetone at  $-10^{\circ}\text{C}$ . with gentle mechanical stirring and centrifuged for 15 min.

*Stage V.* The supernatant is discarded; the residue removed and placed in a vacuum desiccator over concentrated c.p.  $\text{H}_2\text{SO}_4$ . The material should turn white prior to storage at  $-14^{\circ}\text{C}$ . Such a white product is readily powdered and is found to be stable in the dry state. If the drying is not carried out carefully, a brown unstable gum will result.

This stable powder is termed the "Active Alcohol Precipitate" (AAP) and has an activity (Table II) of 2.4 L.U./mg. K.N. and a lipase value of 200 which represents approximately a sixfold concentration and purification over the original starting material. From 30 g. of starting material, approximately 1 g. of this material can be obtained (13).

### Scheme II

It has been found that a suspension of the AAP in water could be clarified by filtration to yield a clear solution of greater activity measured on the basis of T.S. and K.N. As a result, this method served as the initial step.

*Stage I.* Five g. of AAP (L.V. 2.00, 2.4 L.U./mg. K.N.) is suspended in 100 ml. of distilled water in a K. M. blender (low speed) for 1 min. at room temperature. The mixture is allowed to stand for 15 min. more at the same temperature and then centrifuged for 30 min.

# SCHEME II

## Isolation of FLB Lipase

Stage I

Active alcohol precipitate  
(L.V. 200, 2.4 L.U./mg. K.N.)

1. Suspend in distilled H<sub>2</sub>O in a blender for 1 min. at room temp.
2. Let stand at room temp. for 15 min.
3. Centrifuge 30 min.

Stage II

Residue (discard)

Supernatant

1. Cool to 0°C. and strain through a double layer of muslin.
2. Filter through thin layer of amphibole with the aid of suction.
3. Dialyze vs. distilled H<sub>2</sub>O at 3°C. for 24 hr.
4. Add the dialyzed soln. to 3 vols. of 95% alc. at -50°C.
5. Centrifuge 20 min.

Stage III

Residue

Supernatant (discard)

1. Freeze-Dry.
2. When dry its activity is:  
L.V. 365, 6.4 L.U./mg. K.N.

The activity of the starting material was found to vary over 50% between various batches obtained at different periods of growing of the FLB.

*Stage II.* The residue, which has only slight activity (Table II), is discarded. The supernatant is cooled to 0°C., rapidly strained through a double layer of muslin, and then filtered through a Büchner funnel containing a No. 1 Whatman paper layered with several centimeters of amphibole, that has been previously washed with distilled water. The filtrate (supernatant step 2, in Table II) obtained is more active than the original AAP (Scheme I, Stage V, Residue, Table III) in terms of T.S. and K.N. The entire operation is carried out at ice-bath temperatures.

The completely clear filtrate is then placed into cellulose nitrate bags and dialyzed against 3 l. of distilled water at 3°C. for 24 hr. (changing the water about 5 times during this period). At the end of this time, the activity of the dialyzed solution (L.V. 281, 4.6 L.U./mg. K.N.) was identical to that of the undialyzed control kept at the same temperature. This indicates that no highly dissociated prosthetic group is present in the enzyme.

The dialyzed solution is then added slowly with stirring to 3 volumes of 95% alcohol previously cooled to  $-50^{\circ}\text{C}.$ , not allowing the temperature to rise above  $-10^{\circ}\text{C}.$  The whole is quickly centrifuged for 20 min.

*Stage III.* The supernatant is discarded and the residue freeze-dried. In this way, a very fine dry and white powder is obtained that has a L.V. of 365 and 6.4 L.U./mg. K.N. (Table II), which represents a 15-fold concentration and purification over the original starting material (*i.e.*, ethyl-ether defatted FLB).

### VIII. Instability of FLB Lipase

Throughout the isolation studies, it had been noticed that aqueous suspensions or extracts of the FLB lipase were extremely unstable. Some research workers are of the opinion that this phenomenon is due to proteolysis, probably brought about by trypsin (21, 25, 41). In fact, Platt and Dawson (41) believed that lipases are stable in over 30% glycerol, since trypsin is inactive under such conditions. However, Singer (47) claims that in the case of wheat germ lipase the instability is due to the oxidation of essential  $-\text{SH}$  groups. Evidence is presented in this paper that the aqueous solutions of FLB lipase are destroyed by proteolysis alone, and that the destructive agent has no essential  $-\text{SH}$  groups.

With FLB lipase, the stability of the enzyme in water mounts with increased purification, indicating the removal of some "inactivator."

TABLE III

*Stability of Aqueous Preparations of FLB Lipase at Various Temperatures*

Material tested	Incubation period	Incubation temperature	Loss in activity from control
	hr.	$^{\circ}\text{C}.$	%
Aqueous suspension of AAP <sup>a</sup>	3	37	100
	24	28 <sup>b</sup>	100
	24	20	90
	24	3	50
Clear filtrate of AAP <sup>a</sup> suspension	3	37	100
	24	28 <sup>b</sup>	80
	24	20	70
	24	3	0
	48	3	50

<sup>a</sup> AAP = Active alcohol precipitate.

<sup>b</sup> Room temperature.

Thus a water-suspension of the AAP when stored at 3°C. for 24 hr. (under c.p. toluene) was found to lose 48–50% of its activity; whereas a clear preparation of the same suspension, obtained as in Scheme II, was found to be completely stable under similar conditions.

A comparison of the stability of these two preparations at various temperatures is presented in Table III.

The activity of the controls was determined at zero time. It was observed that, in preparing the blanks for the determination of the activities of the preparations of the clear filtrate of the AAP suspension, the greatest amount of coagulum, on heating, was obtained with the solutions having the most activity (*e.g.*, the control or sample kept at 3°C. for 24 hr.) and the least with the solutions having least activity (*e.g.*, a sample kept at 37°C. for 3 hr.). This is a good indication of the occurrence of the proteolytic destruction of the lipase.

If either of the above two preparations are dialyzed against distilled water at 3°C., it will be noticed that they contain dialyzable nitrogen (17) as shown by a drop in per cent K.N. of the dialyzed solutions as compared with undialyzed controls. This indicates either the presence of dialyzable nitrogen in the original preparations or the liberation of amino acids during dialysis, probably brought about by proteolysis. At the same time it will also be noticed that there is an increase in activity on the basis of T.S. and K.N.

### *XI. Electrophoresis Studies*

Some electrophoresis studies were attempted with preparations of lipases by Boissonas (5). However since no patterns were presented the data cannot be evaluated.

In order to locate the enzyme in a particular fraction, the study of patterns with a highly purified FLB lipase preparation was started. It was further desired to use electrophoresis in order to obtain evidence as to whether or not the lipase is destroyed by proteolysis.

The preparation chosen for this investigation was our most active FLB lipase material (Scheme II, Residue III). This had been obtained by dry-freezing the clear lipase solution immediately after the 24-hr. dialysis period and is denoted as the "Active Preparation." An "Inactive Preparation" was obtained by taking the same clear solution of lipase immediately after dialysis and placing it at 37°C. for 3 hr. with constant shaking. These conditions completely inactivated the lipase.

This material was then freeze-dried as in the case of the "Active Preparation."

When testing for activity, as before, on heating the "Active Preparation" in order to prepare the blank, a large amount of coagulum was obtained whereas practically none was present on heating the inactive material. Again, this is evidence of proteolysis.

To prepare a highly concentrated lipase, as recorded in the isolation procedure, precipitation of the lipase is carried out in ethyl alcohol after dialysis. Such treatment with alcohol was eliminated for these studies in order to minimize differences that might occur in the amount of substances soluble in the alcohol supernatant between the preparations subjected or not subjected to incubation at 37°C. for 3 hr.

The conditions and apparatus used in these studies have been described elsewhere (29). The electrophoresis patterns of these two preparations in phosphate and barbiturate buffers at 0°C. are presented in Fig. 3. An analysis of these patterns is recorded in Table IV.

The results indicate that two main components are present at this point in the concentration and purification of the FLB lipase. At least two proteins are noted in the ascending portion of pattern 1 (fast fraction). Furthermore, the fact that the fast fraction of both preparations spreads over a large area in the barbiturate buffer indicates the presence of several electrophoretic constituents which are extremely difficult to separate. This is emphasized also by the fact that it is known

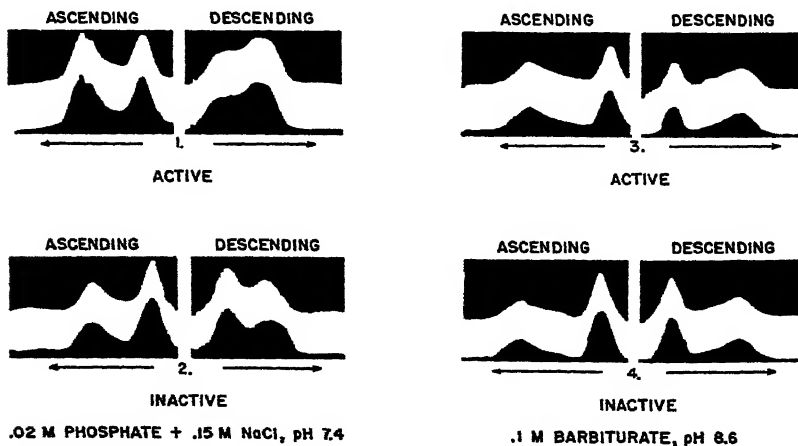


FIG. 3. Electrophoresis Patterns of Active and Inactive Lipase Preparations.

TABLE IV

*Analyses of Electrophoresis Patterns of Active and Inactive Lipase Preparations*

Buffer	Material	Pattern no	Percent total pattern area		Mobility $\mu \times 10^{-5}$	
			Fast	Slow	Fast	Slow
0.02 M Phosphate — 0.15 M NaCl, pH 7.4	Active	1	58.6	41.4	4.50	0.45
	Inactive	2	46.4	53.6	4.50	0.00
0.1 M Barbiturate, pH 8.6	Active	3	63.4	36.6	3.90	0.10
	Inactive	4	48.3	51.7	5.20	0.49

<sup>a</sup> Average of ascending and descending mobilities.

that the "active preparation" contains in addition to lipase, amylase (11) and the destroying proteolytic enzyme. This preparation has been found capable of digesting casein.

Inspection of Table IV, clearly brings out the case of the lipase destruction; *i.e.*, proteolysis. In both buffers, when inactivation occurs, the per cent total pattern area of the fast component decreases and that of the slow one increases; this would be expected were amino acids and peptides produced by proteolysis during inactivation (27). This means, also, that the lipase occurs in the fast fraction, comprising only a small portion of it. As noted above, this fraction consists of several components that are electrophoretically inseparable.

### X. Inhibition Studies

It was desirable to find out whether the FLB lipase contained essential —SH groups as a further possible explanation for the instability of the enzyme in water.

All the determinations were carried out quantitatively and qualitatively, *i.e.*, using the triacetin method and the Huggins' color test, respectively. The reagents (40) applied were as follows:

Reagent	Concentration
1. Potassium ferricyanide	$2 \times 10^{-3} M$
2. Iodoacetic acid	$5 \times 10^{-3} M$
3. <i>p</i> -Chloromercuribenzoic acid	$1 \times 10^{-3} M$
4. Glutathione	$1 \times 10^{-2} M$
5. Cysteine-HCl	$1 \times 10^{-2} M$
6. Thioglycolic acid	$1 \times 10^{-2} M$

1. *Reactivation Experiment.* Using Reagents 4, 5, 6, an attempt was made to reactivate the *inactive* lipase which had been subjected to electrophoresis studies (*vide* Fig. 3, patterns 2 and 4). This material could not be reactivated. Consequently, we can say that inactivation is not due to the oxidation of essential —SH groups but most probably due to proteolysis. However, it cannot be concluded that the FLB lipase contains no such groups since it could still possess essential —SH groups which could not be detected as essential, after its destruction by proteolysis.

2. *Inhibition Experiments.* Using Reagents 1, 2, 3 (above), we attempted to inhibit the *active* lipase preparation which had been subjected to electrophoresis studies (*vide* Fig. 3, patterns 1 and 3). However, under the conditions of the experiment, no inhibition could be detected. It is therefore concluded that FLB lipase, unlike wheat germ lipase (47), contains no —SH groups essential for its activity.

3. *Stabilization Experiment.* Knowing that the FLB lipase had no essential —SH groups, we then tried to stabilize the active lipase preparation used above, by the addition of *p*-chloromercuribenzoic acid. This would take place only if the destroying proteolytic enzyme contained essential —SH groups which could react with this reagent.

Thus the lipase preparation was divided into two portions, one of which was inactivated by placing it at 37°C. for 3 hr. with constant shaking, the other portion being treated the same way but in the presence of  $1 \times 10^{-3} M$  *p*-chloromercuribenzoic acid. At the end of this time *both* preparations were found to be *inactive*. We can therefore conclude that the destructive agent is devoid of essential —SH groups.

As mentioned previously, the literature indicates that trypsin is the agent that destroys the lipases. According to Barron (46), *p*-chloromercuribenzoic acid does not inhibit trypsin at a concentration of  $1 \times 10^{-3} M$ . Our evidence appears, therefore, to corroborate the consideration that trypsin *might* be the destructive agent.

4. *Activation Experiment.* Using Reagents 4, 5, 6, their effect on an *active* FLB lipase preparation was tested. Thus using the same enzyme preparation employed in the inhibition experiments above it was found that these agents have neither an activating nor inactivating effect. It is, therefore, concluded that the FLB lipase contains no —S—S— or —SH groups essential for its activity.

5. *Use of Crystalline Soy Bean Trypsin Inhibitor.* The accumulated evidence points toward trypsin as being the lipase destructor. To confirm this, crystalline soy bean trypsin inhibitor (five-times recrystallized and salt-free) was used (38).

The lipase solution used previously in the inhibition, stabilization, and activation experiments (described above) was used in this study. The solution contained approximately 20 mg. T.S./ml. Forty mg. anti-trypsin was suspended in 9.5 ml. water and 0.5 ml. of McIlvaine buffer (pH 7.0) was added to effect solution (0.4% solution). A portion of the lipase solution was diluted 1:1 with water containing buffer (made up

by mixing 9.5 parts of water to 0.5 parts buffer), and was kept at 6°C. for 30 min. At the end of this time, the preparation was incubated at 37°C. for 3 hr. Another portion of the lipase solution was diluted 1:1 with the antitrypsin solution and treated in a similar way. At the end of this time both solutions were tested for activity and found to be completely negative. This evidence seems to indicate that trypsin, contrary to the above recorded observation, is not the destroying agent. If it were, the lipase solution containing the antitrypsin should still display lipolytic activity.

### *XI. Comparison of FLB Lipase with Steapsin*

It was of interest to compare the activity of a concentrated FLB lipase preparation with commercial pancreatic lipase. Steapsin, in terms of T.S. and K.N. by two methods; *i.e.*, by the triacetin method used throughout our lipase studies and by the newly-developed PVA-Olive oil method (13). The enzyme material was prepared as before (13). A preparation of Steapsin was obtained by treating 200 mg. of commercial pancreatic lipase in a similar way.

In the PVA-Olive oil method (13), the lipase unit is defined as that amount of dry enzyme preparation which liberates acid in amounts equivalent to 1 ml. of 0.05 *N* aqueous NaOH when allowed to act on 10 ml. of PVA-emulsified olive oil (3% by volume) and 5 ml. of McIlvaine buffer at pH 7.0, 37°C. for 4 hr., the final volume being 20 ml. (The lipase value is the number of lipase units g. of dry enzyme preparation.)

The results of these determinations are recorded in Table V.

TABLE V  
*Comparison of the Relative Activities of FLB Lipase and Steapsin*

Method used	Material tested	T S	K N.	L.U./ml.	L.V.	L.U./mg. K.N.
Triacetin	FLB	mg./ml. 23.6	mg./ml. 2.90	3.8	119	1.3
	Steapsin	1.2	0.19	4.5	3750	24.0
PVA-olive oil emulsion	FLB	23.6	2.90	0.4	17	0.1
	Steapsin	1.2	0.19	1.2	1000	6.3



It can thus be seen that steapsin, as obtained commercially, is about 30 to 50 times more active than the concentrated FLB lipase preparation used for the comparison study.

### COMMENTS

It has been pointed out (8) that some parallelism exists between the phenomena observed when esters in monolayers are hydrolyzed by hydroxyl ions and those observed when the enzyme lipase is the catalytic agent. It also of course has been realized for a long time that reactions at interfaces, some of which are of great importance in biology, present peculiar features. Since lipases are insoluble in lipoids, the reaction site is probably limited to interfacial regions involving a lipid-water interface. Thus attempts to study lipolytic action *in vitro* always presents difficulties in establishing artificial conditions as rigid as those prevailing in the natural habitat of these enzymes.

Although triacetin has been used throughout these studies for the determination of lipolytic activity, this has been done for convenience and does not simulate natural conditions. The use of polyvinyl alcohol in lipase determinations (13) is sufficiently emphasized, from a theoretical point of view, if we consider the fact that, in addition to natural fats being so insoluble in water and lipases functioning at a lipid-water interface, the enzymes catalyzing reactions acting on fats are themselves large molecules. The reactions they catalyze are then presumably occurring at the surface of the enzymes; that is, at an enzyme-water interface, and in no sense in a true *solution*. The use of polyvinyl alcohol for the emulsification of water-insoluble substrates provides stable emulsions which seem to present the requisite conditions of lipid-water and enzyme-water interfaces.

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### SUMMARY

1. A method for the quantitative determination of the lipolytic activity of *Fusarium lini* Bolley (FLB) has been presented.

2. Using triacetin as the test substrate, the FLB lipase was found to display optimum activity at pH 7.0 and at 37°C.

3. The lipase of FLB was found to be intracellular, very stable in the dry state, unstable in aqueous solutions and to be soluble in various extracting agents. It does not seem to possess a dissociable prosthetic group.

4. Using dry, ethyl-ether defatted FLB as a starting substance, a method is presented by which a dry, stable powder is obtained which is about 15 times purer and more concentrated than the starting material. This method involves essentially the use of acid and alcohol.

5. The instability of the enzyme in water is probably due to proteolysis. However, its stability in this solvent increases with purification.

6. Electrophoresis studies on a concentrated and purified lipase preparation show the presence of two main components, one of which consists of several inseparable proteins. These studies support the assumption that the enzyme destruction in aqueous solutions results from proteolysis.

7. The FLB lipase contains no —SH or —S—S— groups essential for its activity.

8. The destruction of the lipase is due to proteolysis by an agent that contains no essential —SH groups. This destructive agent is probably not trypsin since the use of crystalline soy bean trypsin inhibitor does not arrest this destruction.

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# The Growth of Phenol-Utilizing Bacteria on Aromatic Carbon Sources

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## INTRODUCTION

The degradation of phenol and related compounds by bacteria has been reported by a number of investigators (1-7), but studies on the influence of chemical structure of various aromatic compounds on the growth of phenol-utilizing bacteria have been generally neglected. During this investigation, 14 organisms were examined to determine their ability to grow on 55 compounds. The large number of compounds upon which these organisms were found to grow reveals their vast potentialities for mineralizing organic matter.

## EXPERIMENTAL

### *Methods and Materials*

*Media.* A chemically defined medium described by Gray and Thornton (3) was used throughout this work. It was of the following composition:  $K_2HPO_4$ , 0.1%;  $MgSO_4 \cdot 7H_2O$ , 0.02%;  $NaCl$ , 0.01%;  $CaCl_2$ , 0.01%;  $FeCl_3$ , 0.002%;  $(NH_4)_2SO_4$ , 0.1%; and phenol, 0.007 M; pH 7.2-7.5. All other compounds used as a carbon source were tested in concentrations of 0.01 M, 0.005 M, and 0.001 M. The components of the basic medium were autoclaved separately and added aseptically to sterile distilled water to give the desired concentrations. Solid medium was made by incorporating the salts in 2% sterile agar. The compounds under test were added without sterilization to the salt-agar medium and adjusted to pH 7.2-7.5 with 0.1 N NaOH.

*Isolation of Organisms.* Approximately 1-g. portions of arable soils were inoculated into 250-ml. Erlenmeyer flasks containing the basic medium without agar plus 0.007 M phenol as the sole source of carbon. After 3 days at 30°C., 0.1 ml. was subcultured to a flask of fresh sterile medium. This procedure was repeated three times. From the third flask, one loopful was streaked on basic medium containing 2% agar and 0.007 M phenol. Representative colonies were picked and maintained as stock cultures on phenol agar slants and stored at 4°C.

### *Estimation of Growth on Test Compounds*

Growth on the compounds tested was determined by the method of den Dooren de Jong (8). The amount of growth observed on agar plates containing the specific carbon compound was compared with the growth observed on the control plates not containing the compound. In most cases no macroscopic growth was observed on the control plates; if growth appeared, then the compound under test was considered positive only when there was distinctly a much greater amount on the agar containing the compound. All plates were incubated at 30°C. for 5 days.

### *Morphological and Biochemical Characteristics of the Bacteria Studied*

All 14 species studied were gram negative, non-acid-fast, non-spore-forming bacteria. They did not hydrolyze starch, liquefy gelatin, nor produce indole or hydrogen sulfide. The Voges-Proskauer and methyl red tests were negative. Carbohydrates were generally not fermented. Morphologically they were small rods, diplococci and a vibrio. Optimum growth occurred aerobically at temperatures from 20–37°C. An alkaline reaction was generally observed in litmus milk. Motility, nitrate reduction, and ammonia production varied among the species.

On the basis of these characteristics three different genera were distinguished (9). Five cultures were classified as species of the genus *Micrococcus*; one culture in the genus *Vibrio*; and eight cultures in the genus *Achromobacter*.

## RESULTS

When the 14 cultures were tested against 55 compounds for their ability to produce macroscopic growth after 5 days incubation at 30°C., they were found to fall into three groups as shown in Table I.

## DISCUSSION

From these results definite correlations can be observed. In general, the organisms were unable to produce visible growth under the conditions tested on saturated ring compounds, diphenyl compounds, or compounds in which the hydroxyl group was substituted by a chloro, amino, or nitro group. The addition of nitro or alkyl groups also tended to prevent growth. Illustrative of this point is the observation that nitrophenols, 2,6-dimethylphenol, diamylphenol, and orcinol do not support growth. The presence of more than one hydroxyl group on the ring made the compound less readily available as is shown in the case of catechol, resorcinol, phloroglucinol, and pyrogallol. These results are in general agreement with the findings of investigators working with species of *Achromobacter* (7) and *Vibrio* (4,6).

TABLE I

*Ability of 14 Phenol-Utilizing Bacteria to Produce Visible Growth on 55 Compounds After 5 Days at 30°C.*

Compounds allowing visible growth of 14 species	Compounds not allowing visible growth of 14 species	Compounds allowing visible growth of some of the 14 species
Benzaldehyde	Saturated ring compounds: Cyclohexane	Benzoic acid (13), <sup>a</sup> Benzyl alcohol (12), Benzenesulfonic acid (12)
Phenyl acetate	Cyclohexanol	Phenylalanine (12)
L(-) Tyrosine	Benzene derivatives: -Cl: Chlorobenzene o-Dichlorobenzene -NH <sub>2</sub> : Aniline p-Phenylenediamine p-Aminodimethyl-aniline -CH <sub>3</sub> : 2-Hydroxy-1,4-dimethylbenzene	p-Hydroxybenzoic acid (11) Salicylic acid (10) Catechol (10) p-Cresol (9) Pyrogallol triacetate (9), Potassium D-isocitrate (5) Benzamide (4), o-Cresol (3) m-Cresol (3) Resorcinol (3), Sulfosalicylic acid (3)
	Phenol derivatives: -NO <sub>2</sub> : o-Nitrophenol m-Nitrophenol p-Nitrophenol 2,4-Dinitrophenol 2,6-Dinitrothymol 3,5-Dinitro-o-cresol 3,5-Dinitrosalicylic acid 2,4-Dinitro-6-phenylphenol Picric acid -CH <sub>3</sub> : 2,6-Dimethylphenol Orcinol Thymol -C <sub>2</sub> H <sub>5</sub> : o-Isopropylphenol -C <sub>6</sub> H <sub>11</sub> : Diamylphenol -OH: Hydroquinone 1,3,5-Phloroglucinol 1,2,3-Pyrogallol Gallic acid -C <sub>6</sub> H <sub>5</sub> : o-Phenylphenol	
	Miscellaneous compounds: Phenyl ether Benzil Diphenylamine Benzidine hydrochloride o-Tolidine Tannic acid p-Toluenesulfonic acid Quinone Lignin Furfural	

<sup>a</sup> Number of organisms giving macroscopic growth on each compound indicated in parentheses.

The presence of a single hydroxyl group or an aliphatic side chain appeared to aid in allowing the ring compound to support good growth; however, it must be emphasized that this property is definitely limited by the number of these groups and by the position in which they are arranged on the ring. Increasing the number of side groups had the tendency to decrease the possibility of growing on the compound. For example, phenyl acetate, which possesses only one acetate radical on the ring, allowed good growth of all 14 organisms whereas the addition of two more acetate radicals, as in pyrogallol triacetate, resulted in growth of only 9 organisms. This point is also shown when the ring contained only one sulfonic group as in benzene sulfonic acid. Here, 12 organisms grew well on the compound; however, when a carboxyl and a hydroxyl group are added to this compound, making it sulfosalicylic acid, only 3 organisms were able to grow well on it. The ability of these organisms to grow on compounds containing a sulfonic group was considered significant in view of the apparent toxicity of this group and the reported failure to support growth of bacteria (7).

The influence of positional substitution in the ring was best illustrated with the cresols and dihydroxyl compounds. With the cresols it was observed that in the para position the compound gave better growth than either the ortho or meta positions. This same preference for para positional compounds was not shown in the use of the dihydroxy compounds. In the dihydroxy compounds, the ortho position (catechol) gave better growth than either the meta (resorcinol) or the para (hydroquinone) compounds.

These results indicate the highly specific nature of the mechanism involved in the degradation of aromatic compounds by bacteria. This conclusion is supported by the observation that minor additions, substitutions, or deletions of simple radicals to the ring greatly alter the ability of the compound to support good growth of the bacteria tested.

#### SUMMARY

Fourteen organisms capable of utilizing phenol were studied for their ability to grow on 55 aromatic compounds. The organisms were classified as belonging to three genera: *Achromobacter*, *Micrococcus*, and *Vibrio*.

On the basis of growth on the 55 compounds, the following generalizations may be made. Saturated ring compounds, diphenyl compounds,

or phenolic ring compounds having a chloro, nitro, or amino group attached do not support visible growth under the test conditions; likewise, increasing the number of side groups tends to decrease the possibility of growth on the compound.

The influence of positional substitution on the ring was observed. With the cresols, the para position allowed better growth than either the ortho or meta position. However, with the dihydroxy compounds the ortho position supported better growth.

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## Studies on the Cyclophorase System. VIII. Oxidation of L-Glutamate

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### INTRODUCTION

The present communication is part of a series devoted to documenting the properties of a complex of enzymes contained within the mitochondrial bodies which we have called the cyclophorase system.<sup>2</sup> Cross *et al.* (1) have shown that glutamate is oxidized in the cyclo-

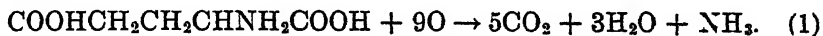
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<sup>2</sup> We have already presented in ten communications a body of evidence that the cyclophorase system is an integrated complex of enzymes which displays some very unusual properties. With the identification of the mitochondrial unit with the structural unit of the cyclophorase system by Lehninger and Kennedy, and Schneider and Potter, as well as in our own laboratory, there are few who now doubt the reality of the cyclophorase system. It has been our purpose and intent to document fully this system (a) with respect to the reactions catalyzed, (b) with respect to the enzymes which implement these reactions, and (c) with respect to the mechanisms by which these reactions take place. Since the cyclophorase system encompasses practically all the principle oxidizing enzymes of the cell, it is of course inevitable that the documentation of this system will cover the same ground that has been covered when some of the individual enzymes which comprise this complex were first described. Thus far we have considered in detail the complete oxidation of pyruvic acid and fatty acids in the cyclophorase system. Naturally we did not discover any important new reactions in these studies. We merely demonstrated that a well-defined complex of enzymes, prepared by a standard method, could carry out the entire citric acid cycle, just as described by Krebs and others, working with whole homogenates, and could carry out the complete oxidation of fatty acids as described by Leloir and others. Our objective has been primarily to document the scope and properties of a system rather than lay claim to the discovery of new reactions. Although we have in the main confirmed the reaction mechanisms of Krebs and others, some contribution has also been made to our knowledge of the details of these reactions, to an understanding of how these reactions proceed, and to the elucidation of some of the operational principles.

phorase system and that phosphate esterification accompanies this oxidation. In this communication data are presented which demonstrate: (a) that glutamate is oxidized to completion in the cyclophorase system by way of  $\alpha$ -ketoglutarate; (b) that the enzyme which carries out the oxidative deamination has properties which are not identical with those of the classical glutamic oxidase described by Euler *et al.* (2) and Dewan (3); and (c) that the cyclophorase complex contains the enzymes necessary for aerobic and anaerobic synthesis of glutamate from  $\alpha$ -ketoglutarate and ammonia just as was shown by Krebs and Cohen (4) and Krebs *et al.* (5) in experiments with tissue minces. The oxidation of glutamate by the cyclophorase system has been singled out for special study since by measurement of ammonia production it is possible to study conveniently a one-step oxidation even though more than one step is involved.

#### *Requirements for Oxidation*

L-Glutamate in the presence of the kidney cyclophorase preparation is oxidized to carbon dioxide, ammonia, and water according to the equation:



Additions of magnesium ions, inorganic phosphate, and adenosine-5-phosphate are needed for maximum rate of oxidation. However, even at the reduced rates which obtained when any one of these components was not added, the ratio of ammonia formed to oxygen absorbed corresponded to complete oxidation of the glutamate undergoing oxidation (cf. Table I).

Apart from the above mentioned three components, no other additions are necessary for maximal rate of oxidation of glutamate. Thus diphosphopyridine nucleotide (DPN) in high concentration has no effect whatsoever on the rate of oxidation of glutamate in a freshly prepared kidney cyclophorase preparation (cf. Table II).<sup>3</sup>

#### *Specificity of Substrate*

The oxidase is specific for L-glutamate. The presence of the D-isomer appears to exert an inhibitory effect on the oxidation of L-glutamate.

<sup>3</sup> With a cyclophorase preparation which has been aged for 24–60 hr. at 0° and then washed in cold saline, a stimulation of glutamate oxidation by added diphosphopyridine nucleotide can be observed.

TABLE I

*Components of the L-Glutamic Oxidase System*

The system contained 1.0 ml. of kidney cyclophorase enzyme at the 3rd residue stage, 0.3 ml. of 0.01 *M* adenylic acid, 0.2 ml. of 0.02 *M* magnesium chloride, and 0.1 ml. of 0.1 *M* phosphate buffer of pH 7.3. Final volume 3.0 ml. Alkali in center well, oxygen in gas space; temperature 38°. Time of experiment, 82 min.

Additions	Oxygen uptake	Ammonia production	Ratio O:NH <sub>3</sub>
	<i>μatoms</i>	<i>μmoles</i>	
System	3.1	4.2	
System + 30 <i>μ</i> moles glutamate	96.0	13.2	10.3
System without phosphate	2.4	5.1	
System without phosphate + 30 <i>μ</i> moles glutamate	69.0	11.4	10.6
System without magnesium	3.1	4.4	
System without magnesium + 30 <i>μ</i> moles glutamate	55.0	10.5	8.5
System without adenylic acid	3.0	2.4	
System without adenylic acid + 30 <i>μ</i> moles glutamate	28.5	5.1	9.5

Although there is present in the cyclophorase preparation the D-aspartic oxidase which shows some activity towards D-glutamic acid, nonetheless, under the conditions of the experiment, D-glutamate appears to be completely inactive. L-Aspartate, L-leucine, and DL- $\alpha$ -amino-adipate are not oxidized at all under conditions which permit rapid oxidation of L-glutamate. Glutamine is slowly oxidized by virtue of its conversion to glutamate through the action of glutaminase.<sup>4</sup>

*Product of Oxidation*

In the presence of 0.0017 *M* arsenite, the oxidation of glutamate is largely arrested at the stage of  $\alpha$ -ketoglutarate (cf. Table III), and a

<sup>4</sup> For each mole of glutamine which disappears more than one mole of ammonia is formed. This extra ammonia arises from the oxidative deamination of glutamic acid. The oxygen uptake corresponds with the theory for the complete oxidation of any glutamate which has undergone the first step of oxidation. The rate with which glutamine is hydrolyzed is not sufficiently rapid to maintain a level of glutamate at which the cyclophorase system can be stabilized by active oxidation. In consequence, oxidation of glutamate ceases within a relatively short time while accumulation of glutamate by hydrolysis continues at a linear rate.

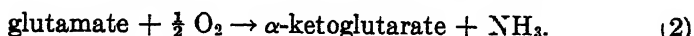
TABLE II

*Effect of Cozymase on L-Glutamate Oxidation*

Conditions as in legend of Table I. Time of experiment, 110 min. Oxygen in gas-space.

Additions	Oxygen uptake $\mu\text{atoms}$	Ammonia production $\mu\text{moles}$
System	5.0	4.7
System + 30 $\mu\text{moles}$ glutamate	83.0	14.9
System + 700 $\mu\text{g.}$ cozymase	6.6	6.3
System + 700 $\mu\text{g.}$ cozymase + 30 $\mu\text{moles}$ glutamate	83.0	15.3

one-step oxidation is approximated according to the equation:



The inhibition of  $\alpha$ -ketoglutarate oxidation by arsenite at the concentration used is not complete. From the fact that the O/NH<sub>3</sub> ratio is greater than 1 while the  $\alpha$ -ketoglutarate/NH<sub>3</sub> ratio is less than 1 it may be deduced that a part of the  $\alpha$ -ketoglutarate formed by oxidation of glutamate is oxidized at least one step further. At higher concentrations of arsenite the oxidation of glutamate is affected as well as the subsequent oxidations.

*Synthesis of Glutamate*

When the kidney cyclophorase preparation is allowed to act on  $\alpha$ -ketoglutarate aerobically in the presence of ammonia, there is observed a disappearance of ammonia and a corresponding increase in L-glutamate (cf. Table IV). That the substance responsible for the appearance of  $\alpha$ -amino nitrogen is L-glutamate can be established by the use of a bacterial decarboxylase preparation specific for L-glutamate. As a control the aerobic oxidation of succinate in the presence of

TABLE III

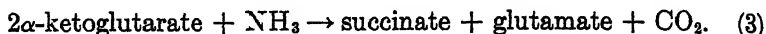
 *$\alpha$ -Ketoglutarate as a Product of Oxidation*

Conditions as in legend for Table I. Duration of experiment 65 min.

Additions	Oxygen uptake $\mu\text{atoms}$	Ammonia production $\mu\text{moles}$	$\alpha$ -Ketoglutarate production $\mu\text{moles}$
System + 0.0017 <i>M</i> arsenite	1.3	3.6	0.3
System + 0.0017 <i>M</i> arsenite + 30 $\mu\text{moles}$ glutamate	9.5	9.5	4.1

ammonia was tested. There was no detectable disappearance of ammonia during the course of the oxidation.

The same synthesis of glutamate can take place anaerobically though the rate is somewhat slower (cf. Table IV). The synthesis is apparently the result of a linked reaction between the reductant of one system ( $\alpha$ -ketoglutarate) and the oxidant of a second system  $\alpha$ -iminoglutarate according to the equation:



The fact that glutamate accumulated during the aerobic oxidation of  $\alpha$ -ketoglutarate in the presence of ammonia suggested that gluta-

TABLE IV

*Ammonia Uptake in Presence of  $\alpha$ -Ketoglutarate*

In experiment *A*, the reaction vessels contained 1.0 ml. of  $\text{R}_3\text{K}$ , 0.3 ml. of 0.01 *M* adenylic acid, 0.2 ml. of 0.05 *M* magnesium chloride, 0.1 ml. of 0.1 *M* phosphate buffer of pH 7.2, alkali in center well, and air in gas space. Duration of experiment 75 min. In experiment *B*, the conditions were the same as for *A* except that the reaction was carried out in evacuated Thunberg tubes.

Conditions	Added $\text{NH}_3$	Added $\alpha$ -keto-glutarate	$\text{NH}_3$ disap-peared	$\text{NH}_3$ disap-peared cor-rected for blank	Glutamate formed
	$\mu\text{moles}$	$\mu\text{moles}$	$\mu\text{moles}$	$\mu\text{moles}$	$\mu\text{moles}$
<i>A</i> Aerobic	30	0	0.3	—	0
Aerobic	30	60	15.8	15.5	13.2
<i>B</i> Anaerobic	20	0	+5.1	—	0
Anaerobic	20	50	8.6	13.7	13.2

mate oxidation should be largely suppressed in the presence of  $\alpha$ -keto-glutarate. For example, in one such experiment 20  $\mu\text{moles}$  of glutamate led to the formation of 10.8  $\mu\text{moles}$  of  $\text{NH}_3$  with an oxygen uptake of 78.5  $\mu\text{atoms}$ . The same amount of glutamate in the presence of 50  $\mu\text{moles}$  of  $\alpha$ -ketoglutarate produced only 2.4  $\mu\text{moles}$  of ammonia although the oxygen uptake was essentially unchanged (86.5  $\mu\text{atoms}$ ).

## DISCUSSION

The glutamic oxidase of the cyclophorase system requires the presence of adenosine-5-phosphate, magnesium ions, and inorganic phos-

phate for full activity but does not require added pyridine nucleotide. In addition esterification of inorganic phosphate accompanies the oxidation. By contrast the isolated Euler-Dewan glutamic oxidase is inactive unless supplemented with either DPN or triphosphopyridine nucleotide (TPN), shows no requirement for adenosine-5-phosphate, magnesium ions, and inorganic phosphate, and finally does not show the phenomenon of oxidative phosphorylation. Elsewhere evidence is presented that the same oxidase is involved, but the state of the oxidase in the cyclophorase system is different from that of the Euler-Dewan isolated oxidase. In the cyclophorase system the fully conjugated glutamic oxidase is involved whereas the enzyme of Euler and Dewan is the dissociated form of the pyridinoprotein.

The cyclophorase complex contains the full complement of enzymes and coenzymes necessary for the complete oxidation of L-glutamate to carbon dioxide and water and for the synthesis of glutamate from  $\alpha$ -ketoglutarate and ammonia.

### EXPERIMENTAL

The cyclophorase suspension was prepared from rabbit kidney and liver as previously described (6). The following methods of analysis were used: ammonia (7),  $\alpha$ -amino nitrogen (8),  $\alpha$ -ketoglutaric acid (9), and glutamic acid (10). The enzyme suspension was prepared for estimation of glutamic acid by freezing and thawing followed by removal of the protein by centrifugation. Ammonia and  $\alpha$ -ketoglutaric acid were estimated in tungstic acid and trichloroacetic acid filtrates, respectively. Ammonia was estimated in the presence of glutamine by nesslerization according to the method of Gentzkow (11). Glutamine was estimated by the method of Krebs (12).

### ACKNOWLEDGMENTS

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### SUMMARY

Some properties of the L-glutamic oxidase of the rabbit kidney and liver cyclophorase system are described.

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## Studies on the Cyclophorase System. IX. Oxidation of L-Alanine

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### INTRODUCTION

In previous communications it was reported that the cyclophorase complex of enzymes catalyzes the complete oxidation of L-proline, L-glutamate, and D-aspartate by way of the citric acid cycle (1-3). The present communication presents evidence that the cyclophorase system contains the full complement of the enzymes and coenzymes necessary for bringing about the complete oxidation of L-alanine.

Braunstein and Bychkov (4) have predicted that the oxidation of L-alanine could be brought about by the alanine-glutamic transaminase collaborating with L-glutamic oxidase.<sup>2</sup> Later Braunstein and Azarkh (6) using minced pig or rat-kidney tissue showed that L-alanine could be deaminated in the presence of  $\alpha$ -ketoglutarate, arsenite, and cozymase. The function of the arsenite was to prevent oxidation of  $\alpha$ -ketoglutarate. In effect their experiment showed that L-alanine can transaminate with excess  $\alpha$ -ketoglutarate to form glutamate which is then deaminated by the Euler-Dewan glutamic oxidase. In the experiments reported below L-alanine is oxidized aerobically in the absence of cozymase or arsenite, and in the presence of catalytic amounts of  $\alpha$ -ketoglutarate which is being constantly generated during the operation of the citric acid cycle. The new features of this demonstration are (1) that the Braunstein-Bychkov prediction of the mechanism of L-alanine oxidation applies to the cyclophorase system where (a)

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<sup>2</sup> Recently Wiss (5) has reported oxidation of L-alanine in minces of liver under conditions which appeared to exclude the participation of transaminase.



only catalytic amounts of  $\alpha$ -ketoglutarate are available, (b) the glutamic oxidase does not require addition of pyridinenucleotide, and (c) all soluble components and enzymes have been removed by thorough washing of the enzyme gel. Of particular interest is the fact that glutamic-alanine transaminase, which is normally considered to be a soluble enzyme, is present as an integral part of the macromolecular particles with which the cyclophorase system is associated.

### *Necessity for Cooxidant*

The kidney cyclophorase preparation at the 3rd residue stage does not catalyze the oxidation of L-alanine unless simultaneously some

TABLE I

### *Necessity for Cooxidant*

The experiments were carried out in manometer vessels containing 1.5 ml. of kidney cyclophorase enzyme (3rd residue), 0.3 ml. of 0.01 *M* adenylic acid, 0.2 ml. of 0.02 *M* magnesium chloride, and 0.1 ml. of 0.1 *M* phosphate buffer of pH 7.2 in a total volume of 3.0 ml. Air in gas space, alkali in center well. Duration of experiment, 77 min. at 38°.

L-Alanine	$\alpha$ -Ketoglutarate	Oxygen uptake	$\Delta$	Ammonia production	$\Delta$	O/NH <sub>3</sub>
<i>μmoles</i>	<i>μmoles</i>	<i>μatoms</i>	<i>μatoms</i>	<i>μmoles</i>	<i>μmoles</i>	
0	0	7.0	—	5.9	—	
50	0	7.3	0.3	5.2	0	—
0	0.5	8.6	—	5.0	—	
50	0.5	9.6	1.0	5.2	0.2	5.0
0	1.0	12.4	—	5.0	—	
50	1.0	15.1	2.7	5.4	0.4	6.7
0	2.5	23.0	—	5.4	—	
50	2.5	64.5	41.5	10.7	5.3	7.8
0	5.0	37.6	—	5.3	—	
50	5.0	83.5	45.9	12.0	6.7	6.8
0	10.0	70.0	—	5.3	—	
50	10.0	117.5	47.5	12.4	7.1	6.7
0	15.0	89.5	—	4.7	—	
50	15.0	121.5	32.0	10.3	5.6	5.7

TABLE II

*Members of the Citric Acid Cycle as Cooxidants for  
Oxidation of L-Alanine*

Each manometer contained 1.5 ml. of kidney cyclophorase (R K), 3  $\mu$ moles of adenylic acid, 4  $\mu$ moles of magnesium chloride, and 10  $\mu$ moles of phosphate (pH 7.2, in a total volume of 3.0 ml. Air in gas space, alkali in center well. Duration of experiment, 70 min. at 38°C.

Additions	Oxygen uptake	$\Delta$	Ammonia production	$\Delta$
	$\mu$ atoms	$\mu$ atoms	$\mu$ moles	$\mu$ moles
A. $\alpha$ -Ketoglutarate (5 $\mu$ moles)	46.0		5.9	
$\alpha$ -Ketoglutarate + L-alanine (50 $\mu$ moles)	96.0	50.0	13.9	8.0
Succinate (5 $\mu$ moles)	42.0		4.2	
Succinate + L-alanine (50 $\mu$ moles)	80.0	38.0	13.0	8.8
B. $\alpha$ -Ketoglutarate (5 $\mu$ moles)	43.5		6.5	
$\alpha$ -Ketoglutarate + L-alanine (50 $\mu$ moles)	73.8	30.3	12.1	5.6
Citrate (5 $\mu$ moles)	59.6		6.9	
Citrate + L-alanine (50 $\mu$ moles)	84.0	24.4	12.3	5.4
Fumarate (5 $\mu$ moles)	50.4		7.0	
Fumarate + L-alanine (50 $\mu$ moles)	79.7	29.3	12.4	5.4

member of the citric acid cycle is undergoing oxidation (cf. Table I). In the bulk of the experiments reported below,  $\alpha$ -ketoglutarate has been selected as cooxidant. The most effective amount of cooxidant lies between 2.5 and 10  $\mu$ moles per flask. Below and above this amount the yield of ammonia is decreased. Any other member of the citric acid cycle can replace  $\alpha$ -ketoglutarate as cooxidant (cf. Table II).

When succinate, malate, or citrate are used as cooxidant, the amount of  $\alpha$ -ketoglutarate present at any time is immeasurably small. Even when  $\alpha$ -ketoglutarate is added as cooxidant the same condition applies after the first 10 min. of the experiment. In the control without alanine, the oxidation rapidly comes to a halt whereas the experimental sample continues for more than 1 hr. Thus as soon as the starting supply of  $\alpha$ -ketoglutarate is exhausted (<10 min.), the concentration of  $\alpha$ -ketoglutarate falls to the level which obtains during the steady state of the citric acid cycle.

*Components for the Oxidation*

Since cooxidation of some member of the citric acid cycle is a prerequisite for oxidation of L-alanine, it would be anticipated that at least the same components as are required for the oxidation of the cooxidant would be essential for the oxidation of L-alanine. Table III shows that in the absence of magnesium ions, adenylic acid, and phosphate ions, the induced oxidation of L-alanine proceeds very slowly if at all.

TABLE III

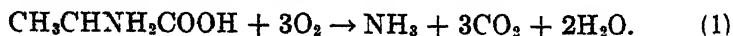
*Requirement for Adenylic Acid, Magnesium, and Phosphate Ions*

Each manometer cup contained 1.0 ml. of kidney enzyme and 5  $\mu$ moles of  $\alpha$ -keto-glutarate in a total volume of 3.0 ml. Duration of experiment, 75 min. Air in gas space.

L-Alanine	Phosphate	Magnesium chloride	Adenylic acid	Oxygen uptake	$\Delta$	Ammonia production	$\Delta$	O/NH <sub>3</sub>
$\mu$ moles	$\mu$ moles	$\mu$ moles	$\mu$ moles	$\mu$ atoms	$\mu$ atoms	$\mu$ moles	$\mu$ moles	
0	0	4	3	37.5	—	6.1	—	—
50	0	4	3	47.5	10.0	8.6	2.5	4.0
0	10	0	3	27.0	—	5.8	—	—
50	10	0	3	28.0	1.0	7.1	1.3	0.8
0	10	4	0	28.5	—	2.8	—	—
50	10	4	0	39.0	10.5	3.9	1.1	9.5
0	10	4	3	37.7	—	5.9	—	—
50	10	4	3	72.7	35.0	11.9	6.0	5.8
0	0	0	0	2.4	—	2.0	—	—
50	0	0	0	3.1	0.7	2.4	0.4	1.7

*Completeness of Oxidation*

It follows from the data of Tables I and III (ratio O:NH<sub>3</sub>) that the oxidation of L-alanine proceeds according to the equation:



In a large series of experiments conducted with air in the gas space, the observed O:NH<sub>3</sub> ratio has been found to approach closely the theoretical value of 6.

*Specificity of Substrate*

D-Alanine is oxidized about one-fifth as rapidly as L-alanine, an oxidation which probably is referable to the traces of the D-amino acid oxidase of Krebs still present in the kidney cyclophorase preparation, at the 3rd residue stage. N-Methylalanine is barely oxidized if at all.

*Inhibitors*

The necessity of a cooxidant to prime the oxidation of L-alanine has analogies with the sparking of fatty acids by members of the citric acid cycle (7). Since 2,4-dinitrophenol and gramicidin both prevent fatty acid oxidation although without effect on the oxidation of  $\alpha$ -ketoglutarate, it was possible that these two reagents would have comparable

TABLE IV

*Effect of Gramicidin and 2,4-Dinitrophenol on Oxidation of L-Alanine*

Each manometer cup contained 1.5 ml. of kidney enzyme (R K), 5  $\mu$ mols of  $\alpha$ -keto glutarate and the usual additions. Duration of experiment 95 min.

L-Alanine	Inhibitor	Final concentration	Oxygen uptake	$\Delta$	Ammonia production	$\Delta$	Inhibition
			$\mu$ atoms	$\mu$ atoms	$\mu$ mols	$\mu$ mols	per cent
Expt. A 0	0	—	35.3	—	5.0	—	—
50	0	—	36.5	48.2	12.0	7.0	—
0	Gramicidin	0.011%	28.1	—	4.5	—	—
50	Gramicidin	0.011%	30.6	2.5	5.7	1.2	83
Expt. B 0	0	—	33.5	—	3.4	—	—
50	0	—	79.5	46.0	10.0	6.6	—
0	2,4-Dinitrophenol	$1.7 \times 10^{-4} M$	28.7	—	3.4	—	—
50	2,4-Dinitrophenol	$1.7 \times 10^{-4} M$	51.0	22.3	7.0	3.6	45
0	2,4-Dinitrophenol	$3.3 \times 10^{-4} M$	27.6	—	3.9	—	—
50	2,4-Dinitrophenol	$3.3 \times 10^{-4} M$	49.0	21.4	6.7	2.8	58
0	2,4-Dinitrophenol	$6.7 \times 10^{-4} M$	33.2	—	4.3	—	—
50	2,4-Dinitrophenol	$6.7 \times 10^{-4} M$	44.6	11.4	6.0	1.7	74
0	2,4-Dinitrophenol	$1.7 \times 10^{-4} M$	29.6	—	4.7	—	—
50	2,4-Dinitrophenol	$1.7 \times 10^{-4} M$	37.9	8.3	5.4	0.7	89

effects on the oxidation of L-alanine induced by  $\alpha$ -ketoglutarate. The data of Table IV shows that the concentrations of dinitrophenol found necessary to suppress completely L-alanine oxidation are considerably higher than are needed to abolish fatty acid oxidation (*ca.*  $3 \times 10^{-5}$  M).

### Mechanism of Oxidation

Below a critical concentration of enzyme, oxidation of L-alanine falls off markedly even though  $\alpha$ -ketoglutarate is undergoing oxidation at a significant rate. This departure from linearity is hardly compatible with the view that the cyclophorase system contains an L-alanine

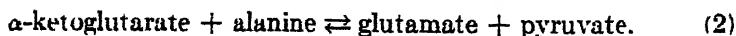
TABLE V  
*Glutamic-Alanine Transaminase*

Experimental vessels contained 1.0 ml. of enzyme, 0.5 ml. of 0.1 M phosphate buffer of pH 7.2, and 0.1 ml. of 0.025 M arsenite in a total volume of 3.0 ml. Time of experiment 5 min. at 33° under anaerobic conditions.

	Pyruvate	L-Glutamate	L-Alanine	$\alpha$ -Ketoglutarate	Final pyruvate	$\Delta$ Pyruvate
	$\mu$ mols	$\mu$ mols	$\mu$ mols	$\mu$ mols	$\mu$ mols	$\mu$ mols
Expt. A						
1	42	0			40.4	1.6
2	42	100			25.6	16.4
Expt. B						
1			100	0	0.8	0
2			100	40	20.9	20.0
3			0	40	2.6	1.7
4			0	0	0.9	—

oxidase which acts directly upon L-alanine; nor does it support the assumption that the oxidation of L-alanine is sparked by  $\alpha$ -ketoglutarate in the same way as the oxidation of fatty acids.

The cyclophorase preparation of rabbit kidney at the 3rd residue stage contains considerable amounts of the glutamic-alanine transaminase (*cf.* Table V) which catalyzes the following reversible reaction:



The formation of pyruvate from alanine or the disappearance of pyruvate in the presence of glutamate can be demonstrated under anaerobic

conditions and it can further be shown that ammonia neither appears nor disappears in these reactions. The rate of this transamination greatly exceeds the rate at which L-alanine undergoes oxidation in the cyclophorase system. Thus the transaminase is present in a concentration adequate to account for the observed rate of oxidation of L-alanine.

According to this interpretation the function of the cooxidant is to provide  $\alpha$ -ketoglutarate which then transaminates with alanine according to Eq. 2. Both pyruvate and glutamate are then burned to completion by way of the citric acid cycle. Once the transamination reaction has begun, the operation of the citric acid cycle insures a level of  $\alpha$ -ketoglutarate adequate to maintain a steady oxidation of L-alanine.

### EXPERIMENTAL

For details, compare previous paper by Still *et al.* (2).

#### Chemicals

The sources of the following chemicals were: L-alanine (Bios Laboratories); D-alanine (gift of Professor K. P. Link); N-Methylalanine (Farchan Laboratories); L-glutamic decarboxylase (gift of Professor R. Burriss); and D-glutamic acid (gift of Dr. J. P. Greenstein).

### ACKNOWLEDGMENTS

This investigation was supported by a grant from the Commonwealth Fund. We are grateful to Mrs. Irene Rechnitz for her assistance with some of the estimations.

### SUMMARY

The conditions and mechanism for the complete oxidation of L-alanine in the cyclophorase system have been studied.

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## Penicillin-Protein Complexes

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### INTRODUCTION

Several investigations have been made of the binding of penicillins by plasma proteins, particularly in connection with the pharmacology of this class of antibiotic. Though the first investigators (1, 2) could detect no effect of human serum on the activity of penicillin, it was soon found by Bigger (3) that bioassays gave low results in the presence of serum. Shortly thereafter, McDermott and Nelson (4) reported that penicillin was bound by some constituent of whole serum, and Chow and McKee (5) described the formation of penicillin-albumin complexes. More detailed studies of the effect of protein binding on the activity of various penicillins were carried out subsequently by Tompsett, Shultz, and McDermott (6, 7), by Eagle and Tucker (8), and by Suter (9).

The present investigation has attempted to extend the fundamental biochemical aspects of preceding studies by consideration of some of the factors which favor formation of penicillin complexes with serum proteins and by extension of the types of protein examined. The results with serum albumin indicate that its complexes with penicillin are similar in character to those formed with simple anions. Spectroscopic manifestations of interactions of samples of crystalline penicillin with cytochrome c have been observed also, but more detailed investigation indicates that these optical changes are associated with the formation or the presence of penicillenic acid.

### EXPERIMENTAL

The extent of binding of each penicillin by serum albumin was determined quantitatively by the differential-dialysis technique described in detail previously (10, 11). Cellophane bags were prepared

from Visking sausage casing and filled with a measured amount of the protein solution. The bag was immersed in a solution of the penicillin contained in a suitable tube which was mounted on a mechanical shaker and placed in an ice bath maintained at 0.0°C. or in a cold room at 5.5°C. Shaking was carried out from 6–18 hr. Preliminary experiments indicated that 6 hr. were adequate for the attainment of equilibrium.

At the end of the equilibration interval, the bag was removed from the tube and the external solution analyzed for the antibiotic. For the heptyl, benzyl, and *p*-hydroxybenzyl penicillins, the iodometric procedure of Alicino (12), as modified by Mundell (13, 14), was used for analysis. Calibrations were run with each sample of penicillin. For the azopenicillins, the color of the substance itself afforded a convenient method of analysis.

Binding experiments with cytochrome *c* were carried out on a semi-micro scale in view of the difficulty involved in obtaining large quantities of the protein. A small dialysis-equilibrium apparatus was devised which utilized cellophane bags of  $\frac{1}{4}$ -in. diameter. Bags containing 1.5 ml. of protein solution were suspended horizontally in a tube containing 3.0 ml. of buffer and antibiotic. Although the thickness of the wall of the cellophane was somewhat greater than that used in the larger apparatus (10, 11), dialysis equilibrium could still be attained in 6 hr.

Of the samples of benzylpenicillin,<sup>1</sup> the first was the pure sodium salt with a potency of 1630 units/mg., and the second was crystalline material from plant production. Heptylpenicillin, *p*-hydroxybenzylpenicillin, and  $\Delta$ - $\beta$ -pentenylpenicillin were obtained from the Antibiotics Study Section of the National Institutes of Health.<sup>2</sup> Two azopenicillins were obtained from Dr. F. H. Stodola of the Northern Regional Research Laboratory, U. S. Dept. of Agriculture.<sup>3</sup>

<sup>1</sup> These samples were obtained through the courtesy of Dr. D. W. MacCorquodale of the Abbott Laboratories.

<sup>2</sup> The original source of the heptylpenicillin was Chas. Pfizer and Company, lot number 7/12/46; that of the hydroxybenzylpenicillin was Commercial Solvents Corp., lot number MS 164835; and that of the pentenylpenicillin was the Upjohn Company, lot number 67-ERH-2.

<sup>3</sup> The crystalline sodium salt of 3-( $\beta$ -naphthylazo)-4-hydroxybenzylpenicillin trihydrate, lot number NRRL 2288-6-A, in the anhydrous state had the following elemental analysis. Found: C, 59.2; H, 4.68; N, 10.6; Na, 4.40. Calculated for  $C_{26}H_{23}O_5N_4SNa$ : C, 59.3; H, 4.42; N, 10.6; Na, 4.37. The sodium salt of 3-(4-bromophenylazo)-4-hydroxybenzylpenicillin, lot number NRRL 1685-44-B, had the following analysis. Found: C, 47.3; H, 3.90; N, 10.1; Br, 14.6; Na, 4.43. Calculated for  $C_{15}H_{20}O_5N_4BrSNa$ : C, 47.57; H, 3.63; N, 10.1; Br, 14.4; Na, 4.14.



The crystallized proteins, bovine serum albumin, ribonuclease, and lysozyme, were purchased from Armour and Company. Samples of cytochrome  $c^4$  of both high and low purities, as assayed by the method of Rosenthal and Drabkin (15), were used, the latter in order to determine the importance of impurities in some of the effects to be described.

All buffers were prepared from reagent grade salts, and pH's were measured with the Beckman glass electrode. Spectra were obtained with a Beckman spectrophotometer, using cells of 1-cm. optical path.

## RESULTS AND DISCUSSION

Since preceding investigations have demonstrated already that penicillins can be bound by serum albumin (5-9), a careful quantitative examination was first made of complexes with this protein. To evaluate binding energies (10), as well as for a consideration of the nature of the bond between protein and antibiotic, it is necessary at the outset to determine the ease of dissociation of the complex.

### *Reversible Nature of Binding of Penicillin by Albumin*

In a typical experiment, summarized in the first part of Table I, to determine whether penicillin-albumin complexes are formed reversibly,  $4.08 \times 10^{-6}$  moles of bovine serum albumin dissolved in 10 ml. of phosphate buffer at pH 6.28 was placed in a cellophane bag which was then immersed in 20 ml. of a solution of benzylpenicillin in the same buffer. The system was allowed to attain equilibrium by shaking at 0°C. At the end of the first equilibrium, the bag was removed from the solution, wiped carefully on the outside to remove all adhering liquid, and then immersed in 20 ml. of a fresh solution of buffer alone. Equilibrium was then permitted to occur a second time.

From measurement of the benzylpenicillin concentration in the solution external to the bag at the end of the first equilibration, and an analysis of the corresponding solution in a control tube with no protein inside the bag, we can calculate the moles of bound as well as free antibiotic within the bag. If the penicillin were bound irreversibly, the free concentration at the end of the second equilibration should be  $\frac{1}{2}$  that introduced ( $3.09 \times 10^{-4}$  M), namely  $1.03 \times 10^{-4}$  M (see Table I). The observed value,  $1.35 \times 10^{-4}$ , is distinctly higher, a qualitative

<sup>4</sup> The sample purchased from the Tremond Company was found, after correction for water content, to be 96.7% cytochrome; that kindly given to us by Dr. G. R. Greenberg of Western Reserve University, prepared by Dr. A. Free, assayed 54% cytochrome.

indication that some bound penicillin must have dissociated from the protein complex.

From the total quantity of penicillin at the start of the second equilibration, and the amount free at the end, the number of moles of antibiotic bound per mole of albumin at the conclusion of the second experiment was calculated to be 0.5 mole, in agreement with the value (0.5) found by equilibrating pure protein with a quantity of penicillin which at equilibrium attains a free concentration of  $1.35 \times 10^{-4} M$  (Fig. 1). Thus the amount of benzylpenicillin bound by albumin is the same whether we start with pure protein and put on the antibiotic, or start with the complex and remove some of the antibiotic.

TABLE I

*Test of Reversibility of Binding of Penicillins by Bovine Serum Albumin*

Antibiotic	Concentration of free penicillin (moles/l.)		Moles bound penicillin per mole of albumin	
	First equilibration	Second equilibration	First equilibration	Second equilibration
Benzylpenicillin	$3.09 \times 10^{-4}$	$1.35 \times 10^{-4}$	0.8	0.5 observed 0.5 calculated
3-( $\beta$ -Naphthylazo)-4-hydroxybenzylpenicillin	$0.57 \times 10^{-5}$	$0.23 \times 10^{-5}$	3.2	3.2 observed 1.5 calculated

A similar reversibility test of 3-( $\beta$ -naphthylazo)-4-hydroxybenzylpenicillin, with a much higher molecular weight, gave less clear-cut results. The concentration of free penicillin in equilibrium with the protein was so much smaller than that in the control tube that corrections for binding by the cellophane bag are more difficult to estimate. It is evident (Table I), nevertheless, that the concentration of free penicillin at the conclusion of the second equilibration,  $0.23 \times 10^{-5} M$ , is slightly greater than the value of  $0.33 \times 0.57 \times 10^{-5} M$ , which one would expect if the complex were completely undissociated. Complete reversibility could not be established, however, because the attainment of equilibrium was very slow, and probably incomplete, due to the small concentration of free penicillin. Instability of the compound prevented use of a longer time interval. It is evident, nevertheless, that the binding is at least partially reversible.

On the basis of these observations, the report of Chow and McKee (5) that albumin-bound penicillin retains its antibiotic properties seems no longer at odds with experiments (6-9) demonstrating the partial loss of antibiotic activity in the presence of serum albumin.

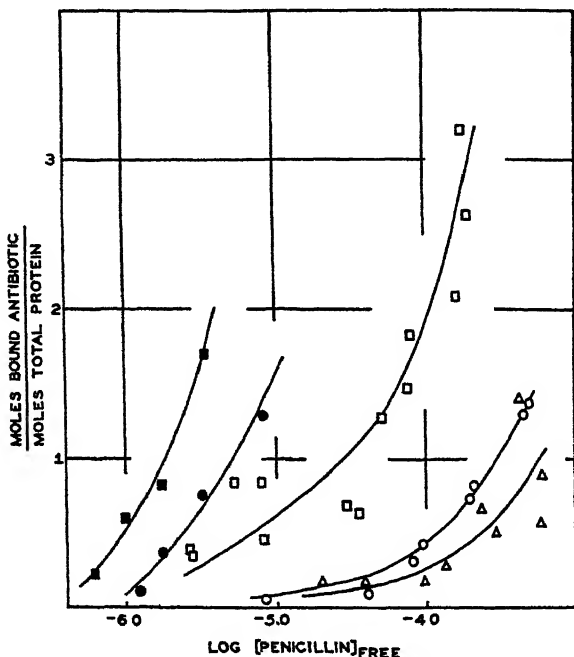


FIG. 1. Binding of penicillins by crystallized bovine serum albumin.  $\Delta$ , *p*-hydroxybenzylpenicillin;  $\circ$ , benzylpenicillin;  $\square$ , heptylpenicillin,  $\bullet$ , 3-(*p*-bromophenylazo)-4-hydroxybenzylpenicillin;  $\blacksquare$ , 3-( $\beta$ -naphthylazo)-4-hydroxybenzylpenicillin.

The isolated complex whose antibiotic activity they examined probably dissociated, releasing free penicillin.

#### *Structure of Antibiotic and Extent of Binding by Albumin*

The extent of binding, at a pH near 6, of each of five penicillins is shown in Fig. 1. This type of plot is convenient for thermodynamic analysis (10, 11). In general the extent of binding increases with increasing molecular size of the antibiotic, as it does in other series of complexes between organic ions and proteins (10, 17, 18). At first

sight, *p*-hydroxybenzylpenicillin may seem to be an exception to this rule. However, it has been observed previously with other aromatic anions that the introduction of a polar hydroxyl group decreases the affinity of the anion for the protein.

Insofar as they overlap, these data confirm the observations of Tompsett, Shultz, and McDermott (6, 7) who also found that binding by albumin increases in the order: *p*-hydroxybenzyl-, benzyl-, and heptylpenicillin.

TABLE II  
*Binding Energies ( $-\Delta F_1^\circ$ ) for Complexes of Bovine Serum  
Albumin with Various Penicillins*

Penicillin	Temperature	pH	$-\Delta F_1^\circ$ cal/mole
<i>p</i> -Hydroxybenzylpenicillin	5.5	6.2	4500
Benzylpenicillin	5.5	6.2	4670
Heptylpenicillin	5.5	6.2	5720
3-( <i>p</i> -Bromophenylazo)-4-hydroxy- benzylpenicillin	0.0	6.2	6630
3-( $\beta$ -Naphthylazo)-4-hydroxybenzyl- penicillin	5.5	5.6	7270

The binding affinities of albumin for the respective penicillins can be expressed thermodynamically as energies of binding. For this purpose, the general theoretical analysis of multiple ion-binding by proteins (10, 11) may be used. In the binding of monovalent anions by serum albumin, electrostatic interactions of successively bound ions are small (10), and hence may be neglected in the present systems. Furthermore, for purposes of comparison within a series of related compounds, it is sufficient to calculate the free energy of binding of the first ion,  $\Delta F_1^\circ$ . The results calculated by methods described previously (10, 19) are assembled in Table II.

The binding energies ( $-\Delta F_1^\circ$ ) of the penicillins parallel the affinities illustrated in Fig. 1. Like binding affinity, the binding energy increases, in general, with increasing molecular weight, except when polar groups, such as  $-\text{OH}$ , are introduced into the molecule.

#### *Changes in Absorption Spectra of Bound Penicillin*

Since the environment of the bound penicillin differs from that of the free anion in aqueous solution, one would expect to find a change in

optical properties of the antibiotic. Of the penicillins studied here, only the azopenicillins, which absorb visible or near-visible light, are convenient for optical examination. The spectra in Figs. 2 and 3 show such a change, which however cannot be attributed from these data to interaction of the protein with any specific group of the antibiotic molecule.

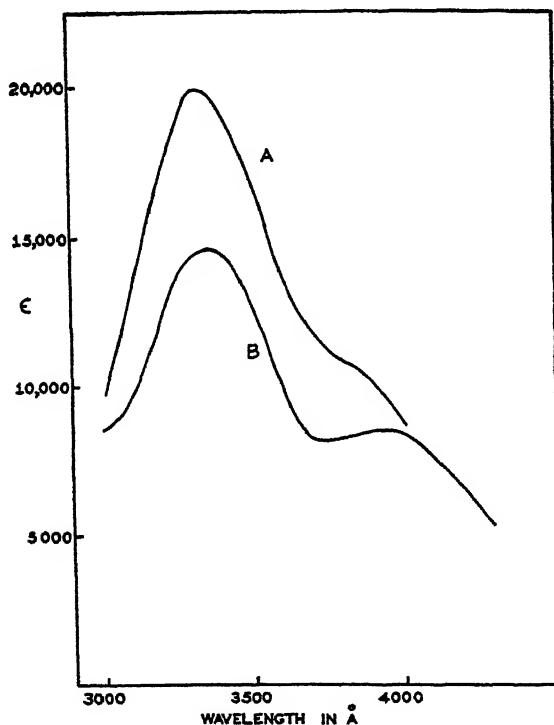


FIG. 2. Absorption spectra of 3-(*p*-bromophenylazo)-4-hydroxybenzylpenicillin: A, in phosphate buffer, pH 6.2; B, in presence of 0.2% bovine albumin in same buffer.

#### *Effect of pH*

Measurements at several values of pH with benzylpenicillin and the naphthylazopenicillin, respectively, showed that the degree of binding was not especially sensitive to pH changes. The extent of binding at various concentrations of benzylpenicillin at pH 5.0 and 7.5 was somewhat less than at pH 6.2; of the naphthylazopenicillin at pH 7.6, somewhat less than at pH 5.6.

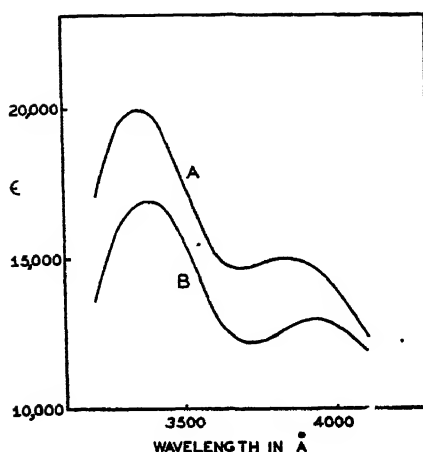


FIG. 3. Absorption spectra of 3-( $\beta$ -naphthylazo)-4-hydroxybenzylpenicillin: A, in phosphate buffer, pH 5.8; B, in presence of 0.2% bovine albumin in same buffer.

### *Effect of Temperature*

Attempts to measure the binding of benzylpenicillin at 25°C. gave erratic results, probably because of the increasing rate of decomposition of the antibiotic with increasing temperature (21, 22). Reasonable reproducibility was obtainable at 15°C. (Table III). Except for the lowest two concentrations, the binding of benzylpenicillin by albumin decreases with increasing temperature. The binding process thus must be accompanied by heat evolution (19). The experimental precision, however, does not warrant the calculation of the enthalpy or entropy change.

TABLE III  
*Effect of Temperature on Binding of Benzylpenicillin  
by Bovine Serum Albumin*

Concentration of Penicillin $\times 10^{-4}$	Moles Bound 5.5°	Penicillin/Mole Protein 15.1°
3.1	0.18	0.24
4.3	0.23	0.34
11.6	0.46	0.35
14.3	0.56	0.38
23.2	0.85	0.66
30.9	1.04	0.52
52.2	1.40	1.07
56.4	1.52	1.01

*Studies with Ribonuclease and with Lysozyme*

Massart, Peeters, and van Houcke (23) report that penicillin inhibits the activity of ribonuclease upon yeast nucleoproteins. Yet Gros and Rybak (24) found no such inhibition. An attempt was made, therefore, to detect complex-formation between this antibiotic and the enzyme. Dialysis-equilibrium experiments showed no decrease in concentration of free antibiotic in the presence of ribonuclease. Thus, within the precision of present analytical techniques, no complex-formation occurs.

Lysozyme, a strongly cationic protein at physiological pH's, is capable of forming complexes with nucleic acids and anionic proteins as well as with several small anions (25). It seemed of interest, therefore, to investigate interactions with the anionic penicillins.

Complex-formation between benzylpenicillin and lysozyme was not detected. This negative result can perhaps be explained by the fact that this anion is bound to albumin with lesser affinity than methyl orange, which is not bound by lysozyme.

The high-molecular-weight naphthylazopenicillin, on the other hand, does interact strongly with lysozyme to give insoluble complexes. Numerous anions can be bound by a single molecule of the protein (Table IV). The behavior of lysozyme with the penicillins, as with other small anions (25), shows that complex-formation does not occur until the ion is of relatively high molecular weight.

TABLE IV

*Complexes of Lysozyme with 3-( $\beta$ -Naphthylazo)-4-hydroxybenzylpenicillin<sup>a</sup>*

<u>Total penicillin</u> <u>Total lysozyme</u>	<u>Free penicillin</u> <u>moles/l. <math>\times 10^{+4}</math></u>	<u>Moles bound penicillin</u> <u>Moles total lysozyme</u>
0.65	0.30	0.42
1.29	0.40	0.84
1.94	0.48	1.23
3.02	0.8	3
4.51	0.7	4
6.05	0.8	5
6.84	0.8	6
12.10	0.9	10
13.69	1.0	13
27.38	1.0	25
44.9	1.0	40
67.3	1.4	60

<sup>a</sup> At pH 6.67.

*Interactions with Cytochrome c*

Spectrophotometric studies indicate some type of interaction between cytochrome c and very pure samples of heptyl-, benzyl-, *p*-hydroxybenzyl-, and  $\Delta$ - $\beta$ - $\gamma$ -pentenylpenicillin, respectively. As is illustrated in Fig. 4, the addition of the penicillin, at a concentration of 0.002 *M* in a phosphate buffer at pH 7.0, to the (ferri) heme protein, 0.00007 *M* in phosphate buffer, results in the production of two horns at 520 and 550  $m\mu$ , superimposed upon the characteristic broad absorption with a peak near 530  $m\mu$  (Fig. 5).

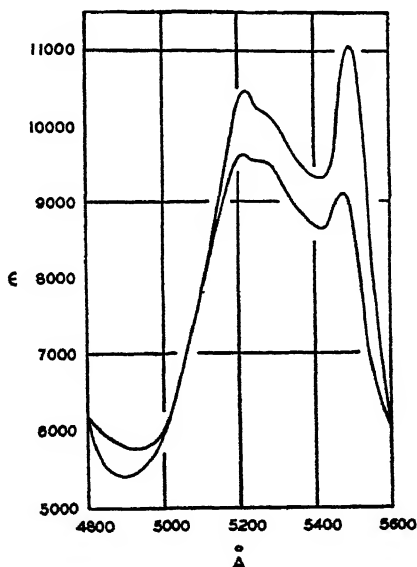


FIG. 4. Spectra of cytochrome c (0.00007 *M*) upon addition of penicillin to a concentration of 0.002 *M* at pH 7.0 in 0.1 *M* phosphate buffer. Lower curve, benzylpenicillin; upper, heptylpenicillin.

The spectra illustrated in Fig. 4 were obtained with the cytochrome c which was 97% pure. To rule out the possibility that the spectroscopic changes were due to hemin impurities, a separate experiment was run also comparing the pure cytochrome c with the sample which assayed 54%. The heights of the horns were much less pronounced than those observed with the 97% cytochrome. The ratio of extinction coefficients at 549  $m\mu$  for cytochrome c plus penicillin and cytochrome c alone was



1.17 for the 97% sample and 1.10 for the 54% sample. It seems unlikely, therefore, that the observed effects are due to spectroscopic changes in hemin impurities.

To determine the effect of possible impurities in the penicillin, a comparison of "plant crystalline" and pure crystalline benzylpenicillin was made, with equivalent concentrations of penicillin. No significant

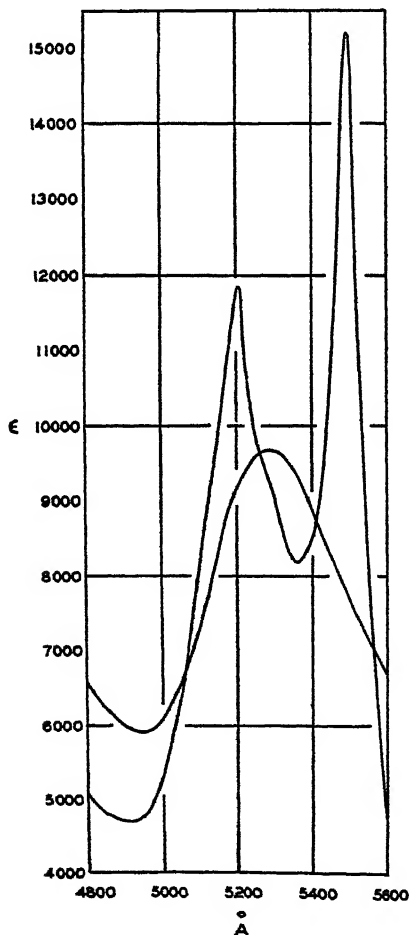


FIG. 5. Reduction of ferricytochrome c (upper curve) at pH 7 by penicillin (heptyl-,  $1.7 \times 10^{-3} M$ , or benzyl-,  $2.2 \times 10^{-3} M$ ) heated to  $100^{\circ}C$ . in acetate buffer (pH 4.6) and neutralized to pH 7. Lower curve, pure ferricytochrome.

difference between the two samples was noted in their effect on the cytochrome spectrum. In the same connection a sample of benzylpenicillin was heated to boiling in a phosphate buffer at pH 7 and the effect of this sample on the spectrum was compared with that of an unheated sample. The results showed no increased effect due to decomposition by heat.

TABLE V

*Effect of Concentration of Penicillin on Optical Absorption of Cytochrome c*

Variety of penicillin	Concentration $\times 10^{-3} M$	Relative $\Delta\epsilon$ at 549 m $\mu$
Benzylpenicillin	2.31	(1.0)
Benzylpenicillin	11.4	5.4
Benzylpenicillin	22.3	11.3
Heptylpenicillin	0.425	(1.0)
Heptylpenicillin	2.16	4.5

The height of the horns developed on the ferricytochrome peak is a direct function of the concentration of penicillin, as has been demonstrated with heptyl- and benzylpenicillin (Table V). In addition it is sensitive to the ionic strength of the medium (Table VI). The dependence on ionic strength is to be expected from the Debye-Hückel theory (26) for complexes between a cation (cytochrome) and an anion; for the electrostatic part of the attraction between two oppositely-charged species would increase if the ionic strength of the solution were decreased.

TABLE VI

*Effect of Ionic Strength on Optical Absorption of Cytochrome c in Presence of Heptylpenicillin<sup>a</sup>*

Ionic strength	$\Delta\epsilon$ at 549 m $\mu$
0.0	3050
0.002	1250
0.2	1240

<sup>a</sup> Concentration =  $2 \times 10^{-3} M$ .

Further indication of electrostatic effects in the interaction of penicillin with cytochrome comes from competition experiments with sodium dodecyl sulfate; these show that heptylpenicillin will not produce its usual effect on the heme protein if dodecyl sulfate is added simultaneously with the antibiotic. Furthermore, the addition of do-

decyl sulfate to the penicillin-cytochrome solution with the characteristic double-horn spectrum is followed immediately by the disappearance of the horns. In both experiments the final concentration of dodecyl sulfate was  $2 \times 10^{-3} M$ . These observations parallel those reported in dodecyl sulfate displacement of simple anions from complexes with serum albumin (16).

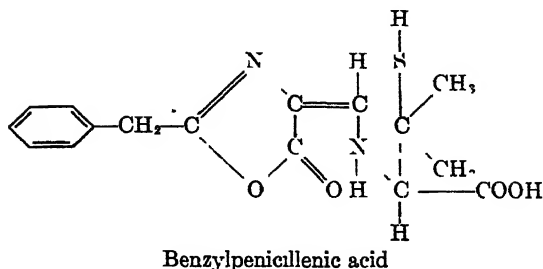
Equilibrium-dialysis experiments to determine quantitatively the extent of binding of heptylpenicillin by cytochrome c showed no detectable complex-formation in the region of  $2 \times 10^{-4}$  to  $2 \times 10^{-3} M$  penicillin. However, since the method of analysis for penicillin is not highly sensitive and since insufficient cytochrome was available to use concentrated solutions, as many as 1-2 moles of antibiotic per mole of protein might be bound and still escape detection.

In view of the reports of metal-catalyzed decomposition of penicillin (27), it seemed worthwhile to examine the possibility that cytochrome might be slowly inactivating the antibiotic. A series of experiments were carried out in which heptylpenicillin was exposed to cytochrome for varying lengths of time and its potency determined by the usual chemical assay (12). At  $0^\circ C$ . and a penicillin concentration of  $1.50 \times 10^{-3} M$ , no significant inactivation of the penicillin occurred. Evidently the horned spectra in Fig. 4 are not those of intermediate complexes in a decomposition process.

The data presented so far are consistent with an interpretation of the penicillin-cytochrome interaction in terms of a complex between the antibiotic and the protein. On the other hand, the shape of the absorption curve (Fig. 4) is very suggestive of a ferrocytochrome c spectrum superimposed on the ferricytochrome c band at  $530 m\mu$ . However, one would not expect benzylpenicillin to possess reducing properties. It seemed necessary, therefore, to examine the possibility that minute impurities in the very pure penicillin samples might be the source of the effect on cytochrome, even though the absence of significant differences between plant grade and pure benzylpenicillin might argue against this explanation.

In view of the ease with which cysteine reduces ferricytochrome c, it seemed appropriate to consider some of the decomposition or rearrangement products of benzylpenicillin which possess mercapto groups, such as penicillamine ( $\alpha$ -amino- $\beta$ -mercaptoisovaleric acid) and benzyl-

penicillenic acid,



each of which can be obtained readily from the penicillin.

When benzylpenicillin was heated in 0.1 *N* HCl at 100°C. for 15 min., the inactivation products, brought up to pH 7, were capable of reducing cytochrome *c* very strikingly. This acid- and heat-treatment forms penicillamine from the antibiotic, and hence any penicillamine in the parent unheated material would act in a like manner. Nevertheless, it seems unlikely that penicillamine was a contaminant of the crystalline penicillins examined since the effect of ionic strength on the reduction of cytochrome was so marked. For a molecule such as penicillamine, with no *net* charge at pH 7, one would not expect interactions with cations to be especially sensitive to ionic strength. On the other hand, with an anion, which penicillenic acid is at pH 7, interactions with cations should be decreased in solution of increasing ionic strength.

Herriott (28) has shown that penicillin heated in an acetate buffer of pH 4.6 at 100°C. for 15 min. forms a product with an ultraviolet absorption maximum at 322  $m\mu$ . Since penicillenic acid shows an absorption peak at 320  $m\mu$  (29), it seems likely that it, or some closely-related substance, is the product of this type of treatment (30). Samples of benzyl- and heptylpenicillin, respectively, were treated thus. Each developed a strong peak at 322  $m\mu$ . Each sample was neutralized to pH 7 and added to a solution of cytochrome *c*. A very substantial reduction occurred, as evidenced by the strong peaks at 520 and 550  $m\mu$  (Fig. 5). Thus if penicillenic acid, or a closely-related compound, were present in the initial penicillin sample, it would produce a reduction of ferricytochrome *c*. As an anion, it would not be attracted as strongly by cytochrome at high ionic strengths as at low. It seems likely, therefore, that the effect of highly purified penicillins on the cytochrome spectrum is due to the presence of minute quantities of penicillenic acid.

The transformation of penicillin into penicillenic acid is catalyzed by metal ions (31), including some, such as zinc and copper, which are found in biological systems. It is possible, therefore, that this transformation could occur under physiological conditions, and that it may play a role in the antibiotic activity of penicillin.

#### ACKNOWLEDGMENT

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#### SUMMARY

Dialysis-equilibrium studies of the binding of penicillins by crystallized bovine serum albumin have led to the following binding energies ( $-\Delta F_1^\circ$ ): *p*-hydroxybenzylpenicillin, 4500 cal./mole; benzylpenicillin, 4670 cal. mole; heptylpenicillin, 5720 cal./mole; 3-(*p*-bromophenylazo)-4-hydroxybenzylpenicillin, 6630 cal./mole; 3-( $\beta$ -naphthylazo)-4-hydroxybenzylpenicillin, 7270 cal./mole. There is a slight decrease in the extent of the binding with an increase in temperature; small variations occur with changes in pH in the region of 5 to 7.5.

No evidence was obtained of complex-formation between penicillin and ribonuclease. Lysozyme forms insoluble complexes with 3-( $\beta$ -naphthylazo)-4-hydroxybenzylpenicillin.

Very pure samples of heptyl-, benzyl-, *p*-hydroxybenzyl-, and  $\Delta$ - $\beta$ - $\gamma$ -pentenylpenicillin produce optical changes in the absorption spectrum of cytochrome c; more detailed examination indicates that these changes are due to interactions with penicillenic acid.

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# **Trans-1,2-Cyclopentanedicarboxylic Acid, a Succinic Acid Analog Affecting the Permeability of the Cell Membrane<sup>1</sup>**

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In the course of investigations on the effect of substituted analogs of succinic and malonic acids on the enzyme systems in the ciliated protozoan *Tetrahymena geleii* (S) results were obtained which indicated that the cyclic analog of succinic acid, *trans*-1,2-cyclopentanedicarboxylic acid, increases the permeability of the cell membrane.

## **METHODS**

Pure, sterile cultures of *Tetrahymena geleii* (S) were grown in 2-l. flasks containing 1 l. of 3% Difco proteose-peptone solution. Inoculations (25 ml.) were made with the aid of sterile 25-ml. pipets. After 72 hr. of growth, the cells were concentrated by gentle centrifugation (750 r.p.m.), washed three times in sterile Hahnert's solution (1) adjusted to pH 5.6, and starved in this solution for 12 hr. At the end of this period, the cells were again centrifuged and resuspended in Hahnert's solution to such a dilution that 1 ml. contained approximately 4 mg. (dry weight) of cells. Two ml. of the washed, starved suspension of cells was added directly to the Warburg vessels. In all cases, the contents of each flask was 3.0 ml. Concentrations of substrates and of the cyclopentane acid are given as final concentration.

In experiments in which homogenates were used, the washed, starved cells were centrifuged to as small a volume as possible and homogenized at 5°C. for 4 min.

Substrate recovery experiments were conducted by incubating living cells with the respective substrates in the presence and absence of the cyclopentane acid. At the end of the experiment, the contents of each flask were transferred to a centrifuge tube, the organisms centrifuged down at a speed of 750 r.p.m. and the amount of substrate remaining in the medium (the supernatant fluid) was estimated. The centrifugation at 750 r.p.m. resulted in no breakage of cells. Acetate was estimated by the colorimetric method of Hutchens and Kass (2); succinate by the method of Krebs (3), and pyruvate by the method of Friedmann and Haugen (4).

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## RESULTS

Table I shows that *trans*-1,2-cyclopentanedicarboxylic acid, in concentrations of 0.04 *M*, increases the oxygen utilization of *Tetrahymena* in the presence of acetate, pyruvate, and succinate (0.02 *M*). The addition of pyruvate and acetate alone results in an increased oxygen utilization of 92% and 71%, respectively, above the endogenous rates. The addition of the cyclopentane acid to the acetate and pyruvate results in an additional increase of 68% and 46%, respectively. The presence of succinate has no significant effect on the oxygen uptake. However, if the cyclopentane acid is added with the succinate there is an increased utilization of 36% above the endogenous value.

TABLE I

*Effect of trans-1,2-Cyclopentanedicarboxylic Acid in the Presence of Pyruvate, Acetate and Succinate, on Oxygen Uptake in T. geleii (S)*

Living cells were used. pH, 5.6; temperature 25.5°C.; gas phase, air

Q <sub>O<sub>2</sub></sub>	Additions
11.5	
11.7	Cyclopentane acid (0.01 <i>M</i> )
11.4	Cyclopentane acid (0.02 <i>M</i> )
11.7	Cyclopentane acid (0.04 <i>M</i> )
11.8	Succinate (0.02 <i>M</i> )
22.7	Succinate (0.02 <i>M</i> ) + cyclopentane acid (0.04 <i>M</i> )
19.7	Acetate (0.02 <i>M</i> )
25.3	Acetate (0.02 <i>M</i> ) + cyclopentane acid (0.04 <i>M</i> )
22.8	Pyruvate (0.02 <i>M</i> )
28.0	Pyruvate (0.02 <i>M</i> ) + cyclopentane acid (0.04 <i>M</i> )

In lower concentrations (0.01 *M*) the cyclopentanedicarboxylic acid has no significant effect on the oxidation of pyruvate, acetate, or succinate. The possibility that the cyclopentane acid is metabolized is precluded by the observation that the compound alone causes no increase in oxygen consumption.

When cell homogenates are used, succinate is rapidly oxidized, as indicated by an increased oxygen uptake of 136% (Table II). The cyclopentane acid inhibits the oxidation of succinate by 47% at an inhibitor-substrate ratio of 1:2; at a ratio of 2:1 the inhibition is 74%.



TABLE II

*Effect of trans-1,2-Cyclopentanedicarboxylic Acid on Succinate  
Oxidation by Cell Homogenates*

pH, 5.6; temperature, 25.5°C.; gas phase, air

Qo <sub>2</sub>	Additions
5.3	
5.4	Cyclopentane acid (0.01 M)
5.5	Cyclopentane acid (0.04 M)
12.5	Succinate (0.02 M)
9.0	Succinate (0.02 M) + cyclopentane acid (0.01 M)
7.2	Succinate (0.02 M) + cyclopentane acid (0.04 M)

These results are as anticipated; *trans*-1,2-cyclopentanedicarboxylic acid, by nature of its structural similarity to succinate, probably competes with the succinate for enzyme surface.

Table III shows that when living cells are used there is an increased rate of disappearance of substrate from the medium in the presence of the cyclopentane acid. In the presence of the acid there is a 50% increase in the amount of acetate removed and a 24% increase in the amount of pyruvate which disappears. Since the addition of succinate results in no significant increase in oxygen uptake (Table I), the observation that living cells do not remove any succinate from the medium is as expected. However, the addition of cyclopentanedicarboxylic acid with the succinate results in the removal of 0.05 mg. of succinate in 30 min. by 8.7 mg. (dry weight) of cells.

TABLE III

*Effect of trans-1,2-Cyclopentanedicarboxylic Acid on Acetate,  
Pyruvate and Succinate Removal from the Medium*

Living cells were used, 8.7 mg. (dry weight). Incubation time, 30 min.; pH 5.6;  
temperature, 25.5°C.; gas phase, air

Substrate disappearance mg.	Additions
0.14	Acetate (0.02 M)
0.21	Acetate (0.02 M) + cyclopentane acid (0.04 M)
0.00	Succinate (0.02 M)
0.05	Succinate (0.02 M) + cyclopentane acid (0.04 M)
0.34	Pyruvate (0.02 M)
0.42	Pyruvate (0.02 M) + cyclopentane acid (0.04 M)

## DISCUSSION

The observation that succinate alone causes no increase in the oxygen uptake of living *T. geleii* (S) indicates that either the organism is not capable of oxidizing this compound or that the cell membrane is not permeable to succinate at the pH level used. The first possibility must obviously be discounted since cell homogenates rapidly oxidize succinate (Table II). Therefore the failure of succinate to effect an increase in oxygen utilization with living cells is probably due to membrane impermeability to the compound. The observation that the addition of *trans*-1,2-cyclopentanedicarboxylic acid, when added to living cells in the presence of succinate, in concentrations twice that of the succinate, results in an increased oxygen uptake, immediately suggests the possibility that the cyclopentane acid affects, in some manner, the permeability of the membrane so as to allow the succinate to enter. The added oxidation of acetate and pyruvate in the presence of the cyclopentane acid may be explained, in like manner, by an increased permeability of the membrane to these compounds; as a result, added amounts of the substrate are capable of making contact with the oxidative enzymes in the cell proper.

The homogenate used was not a true homogenate as the term is generally defined (5). The dilution factor was very small (approximately 1:2) and no additions were made. Therefore, it was, in reality, a tissue mince, closely resembling the living cell with the exception that the cell membrane was destroyed. In such a preparation there is certainly diminution of total enzymatic activity. However, the oxidative enzymes are apparently little affected since the preparation carries on the oxidation of succinate at a fairly high rate. Therefore, the difference in results obtained with living cells and with the tissue mince is probably due to the action of the cyclopentane acid on the one main factor by which the two preparations differed: the cell membrane.

The observation that the rate of disappearance of substrate from the medium is increased in the presence of the cyclopentane acid (Table III) is supporting evidence that the effect of the cyclopentane acid is, in fact, due to an increase in permeability of the cell membrane. These results also tend to rule out such explanations as the cyclopentane acid effecting a phenomenon similar to that exerted by the dinitrophenols.

It seems advisable to define what is meant by the terms "permeability" and "increase in permeability" as used in this report. According to Brooks and Brooks (6), permeability may be defined as "the rate of movement of a substance through the permeable layer under a given driving force." In most cases the driving force is considered to be

a concentration gradient between the two sides of the membrane, the membrane being a porous, lipid protein mosaic. With this view, the rate of penetration of a substance is dependent upon such factors as the concentration gradient, the molecular size of the substance, and its lipid solubility. Thus, agents which increase the permeability rate would be expected to exert such action on the membrane as to change the pore size, or to perhaps affect the substance under investigation in such a way as to change its lipid solubility.

However, the investigations of Cori (7), Wilbrandt and Laszt (8), Lundsgaard (9), and others, have shown that the transfer of sugar across the intestinal mucosa is an "active," enzymatic reaction. In like manner, Davson and Reiner (10) have shown that the transfer of sodium across the membrane of the cat erythrocyte is an "active" process, and Le Fevre (11) has presented evidence that the penetration of glycerol, glucose, and similar substances into the human erythrocyte is also regulated by an "active," enzymatic reaction. The active transport of glutamic acid, glutamine, and histidine across the membrane of *Staphylococcus aureus* has been demonstrated by Gale (12) and it has been shown that the mode of action of penicillin on this organism is to decrease the permeability of the membrane to glutamic acid by inhibiting the metabolic (enzymatic ?) reaction(s) required for transport.

The suggestion that *trans*-1,2-cyclopentanedicarboxylic acid increases the permeability of the membrane of *Tetrahymena geleii* (S) to such substances as succinate, pyruvate, and acetate is not meant to imply that the cyclopentane acid increases the pore size of the membrane, or that it increases the lipid solubility of the compounds investigated. Rather, it is suggested that the acid, in some manner, acts upon the "active transport mechanism" in/on/at the membrane (surface) in such a manner as to accelerate the reactions which govern the rate of penetration of succinate, pyruvate, and acetate.

#### SUMMARY

1. *trans*-1,2-Cyclopentanedicarboxylic acid increases the rate of oxidation of pyruvate and acetate by living *Tetrahymena geleii* (S) as measured by oxygen utilization and by substrate utilization.

2. The membrane of *T. geleii* (S) is impermeable to succinate. The addition of the cyclopentane acid apparently increases the permeability

of the membrane to succinate; in the presence of the acid, succinate is oxidized by the living cells. In like manner, the increased oxidation of acetate and pyruvate in the presence of the cyclopentane acid is due to increased permeability of the cell membrane.

3. *trans*-1,2-Cyclopentanedicarboxylic acid inhibits the oxidation of succinate by cell homogenates.

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## The Action of Electrolytes on Oxalacetic Decarboxylase from *Cucurbita* Seeds

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### INTRODUCTION

The activity of oxalacetic decarboxylase appears to be dependent, to a great extent, on the nature of the ions associated with it in solution. Vennesland, Gollub, and Speck (1), employing an oxalacetic decarboxylase from parsley root, and Plaut and Lardy (2), using an extract of this enzyme from *Azotobacter vinelandii*, have indicated that certain ions used in the buffering system, or as reactants, inhibit the  $\beta$ -decarboxylation of oxalacetic acid. No detailed study has been made, however, of the effect of these ions on the enzyme system, nor has the possible activating or inhibiting effect of the buffering system been ascertained.

Several investigators have indicated that polyvalent cations activate oxalacetic decarboxylase (2, 3). In addition, it has been demonstrated that metallic ions may increase the rate of decarboxylation of oxalacetic acid in the absence of protein (4, 5, 6, 7). Steinberger and Westheimer (8) have indicated that these ions may catalyze the decarboxylation by forming a complex with the carbonyl oxygen and the carboxyl group adjacent to it. Whether the enzyme itself is a metal-protein has as yet not been ascertained.

The present experiments were, therefore, undertaken to observe the effects of various ions on the enzymatic  $\beta$ -decarboxylation of oxalacetic acid.

<sup>1</sup> Part of the data for this study was taken from a thesis presented by Stewart A. Brown in partial fulfillment of the requirements for the Degree of Master of Science in the Graduate School of Michigan State College.

## EXPERIMENTAL

The enzyme preparation used in this study was the crystalline globulin isolated from the seeds of *Cucurbita pepo* according to the method of Vickery *et al.* (9). A similar preparation has been shown to have oxalacetic decarboxylase activity by Vennesland and Felsner (3). Several preparations were tested and the most active sample was used for further study.

In all cases activity was measured in the Warburg apparatus by measuring the volume of carbon dioxide liberated in the decarboxylation of a substrate of oxalacetic acid. A solution or fine suspension of the globulin, diluted 1:320 with water, was pipetted into a Warburg flask, together with a specified volume of acetate buffer (usually 0.5 ml.) of the desired pH.

When working at a pH at which the globulin was soluble, it was found convenient to add the buffer solution to the protein suspension in the correct proportions in a beaker. This resulted in a buffer-protein solution which could easily be pipetted, and the necessity of adding enzyme and buffer separately was avoided. A 0.1-ml. portion of a solution of oxalacetic acid (10 mg./ml. water, prepared by the method of Schneider (10)) was placed in the side arm. Any other materials required in the individual experiments were added to the flask, and water was added to make a total of 3.0 ml. In experiments employing inorganic salts, c.p. or reagent grade chemicals were dissolved in redistilled water. After a 10-min. equilibration period at 30°, zero-time readings were made on the manometer and the reaction was initiated by tipping the flask to mix enzyme and substrate. Since accurate time readings were necessary in these studies, initiation of the reaction in individual manometers was spaced at 30-sec. intervals. Readings were then taken at convenient intervals during the time required for the reaction. All measurements were made in duplicate.

## RESULTS

*Properties of the Enzyme*

An electrophoretic analysis of the globulin preparation indicated that at ionic strength 0.08 and pH 4 in acetate buffer, the protein was essentially homogeneous.

Since it had been shown formerly (7) that metal ions activate the system, an analysis was made of the enzyme preparation for metals.

For this measurement, the globulin was recrystallized twice from a 5% sodium chloride solution in water redistilled in glass and was reprecipitated one more time by dialysis against redistilled water. The globulin was then washed four times with redistilled water. Approximately 7 g. of the purified globulin heated in an oven at about 700° yielded 8 mg. of ash. The composition of the ash was measured spectroscopically, and the presence of several metals was observed. Manganese and iron occurred in what appeared to be more than trace quantities judged from the number and relative intensity of persistent lines. Zinc, cobalt, and nickel were also found but only as traces in the ash. A quantitative analysis of a solution of the ash of the globulin was therefore made for manganese and iron. Using the method of Kun (11) to analyze for manganese it was observed that the globulin contained approximately 0.12 mg. of manganese/100 g. of protein. A spectroscopic analysis of a solution of the ash was also made for iron. It was found that the enzyme contained less than 0.02 mg. iron/100 g. of protein, the lower limit of sensitivity of the method. These experiments indicate the possibility that oxalacetic decarboxylase from *Cucurbita* seeds is a metal-protein, and it may follow that in the presence of added metal ions increased activity of the enzyme, isolated from other sources, results from the formation of this metal-protein complex.

There is a linear relationship between protein concentration and enzyme activity, as illustrated in Fig. 1. Since Vennesland, Gollub, and Speck (1) have shown that the enzyme-catalyzed decarboxylation of oxalacetic acid is first order in substrate, activity was expressed as the first-order rate constant. It will be noted that the difference between the first-order rate constants in the presence of heated and unheated enzyme plotted against protein concentration also gave a straight line. The heat inactivated enzyme was prepared by dissolving the globulin in acetate buffer and heating in a boiling water bath for approximately 2 min. When the globulin was treated in this manner, the heat-inactivated protein did not precipitate and pipetting was thus facilitated.

The activity of the enzyme was measured as a function of pH. It was noted that the globulin was in suspension in buffer more alkaline than pH 4.5, and in solution at a more acid pH. It was also established that the optimum pH range is from 4.0 to 4.5. There is, however, no direct relationship between solubility of the globulin and enzyme activity. At pH 3.5 the enzyme was in solution, but it was no more active than the suspended material at pH 5.5. The pH used for subsequent experi-

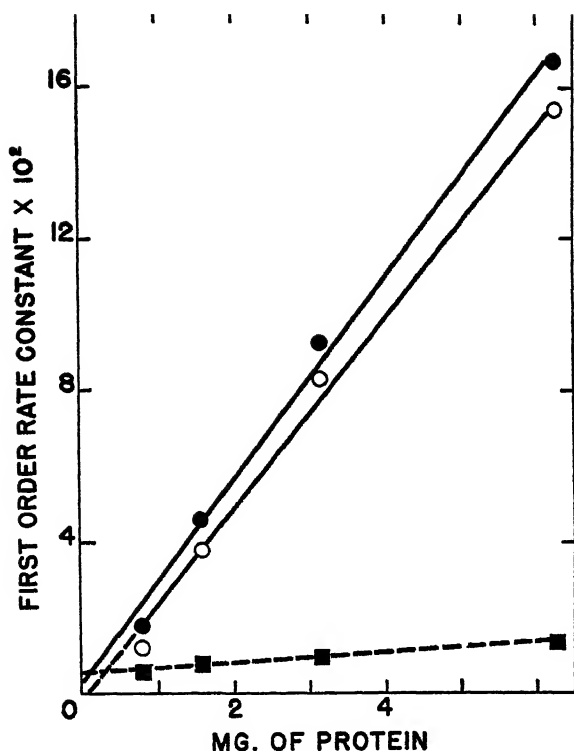


FIG. 1. Relation between globulin concentration and enzyme activity. One-half ml. of 0.1 *M* acetate buffer, pH 4.0 (final *M* = 0.016). One mg. of oxalacetic acid in 0.1 ml.; 1 ml. of enzyme; water to make 3 ml. ● active enzyme; ■ heat-inactivated preparation; ○ represents difference between rate constants in the presence of heated and unheated enzyme.

ments was 4.0 which is close to the optimum under the conditions of the experiment.

#### *Effect of Electrolytes on the Activity of the Enzyme*

The globulin enzyme preparation was soluble in 5% sodium chloride solution, but when dissolved in this concentration of salt solution was found to retain no enzyme activity. As a consequence of this preliminary finding, the influence of various salts on enzymatic activity was measured. The effect of electrolytes which gave only monovalent anions



in solution is shown in Fig. 2. Solutions of the salts were prepared so that after dilution an ionic strength of 0.007 was obtained. The final ionic strength of the sodium acetate used in the buffer was 0.005, and was held constant in this experiment; hence, the total ionic strength<sup>2</sup> of the test system was 0.012. In all cases, when studying the effect of salts, the ionic strength of the control system was raised to 0.012 by using a larger volume of buffer solution. The activity of the enzyme in acetate buffer of ionic strength 0.012 was used as the control. The results are presented as a plot of the logarithm of the micromoles of oxalacetic acid remaining *vs.* time. The fact that the reaction is first order in substrate under the control conditions has already been indicated (1). It can be seen that adding various salts to the buffer has no effect on the order of the reaction in substrate.

It will be noted that there is no direct relationship between ionic strength alone and the enzyme activity in the presence of various individual salts. Activity was similar to the control in the presence of sodium fluoride, whereas in the presence of perchlorates and nitrates, enzymatic decarboxylation was less than 20% of the control in all cases. Activity of the enzyme was about 50% of the control in solutions containing two chlorides. Only one curve is shown for heat-inactivated enzyme because even though there were small differences in the rate of decarboxylation of the substrate in the presence of these salts, the differences were not greater than the differences noted in control heat-inactivated experiments. The values shown are the average of the heat-inactivated enzyme decarboxylation rates, in the control and in the presence of added salts.

Figure 3 shows the effect on the decarboxylation of oxalacetate of electrolytes which yield divalent anions in solution. These salts were again added in such concentrations that, with the buffer, a final ionic strength of 0.012 was obtained. The results were similar to those obtained with monovalent ions. In the presence of magnesium chloride, activity was about 70% of the control, whereas all of the sulfates used in the experiment permitted only slight activity. Calculation of the ionic strength of sodium oxalate, because of the incomplete dissociation

\* The contribution of hydrogen ion and protein to the final ionic strength was considered to be negligible. The ionic strength contribution of the substrate was not negligible, but was constant at the beginning of each experiment. As the reaction proceeded the ionic strength of the substrate changed somewhat but it was not considered feasible to attempt to measure the effect of this changing ionic strength on the rate of the reaction.

of oxalic acid at pH 4.0, gave no more than a close approximation. The ionic strength calculations for citrate, not shown in Fig. 3, were also approximations, but concentrations of citrate greater than were required to give an ionic strength of 0.012 showed no measurable change in activity of the enzyme. Again only one line is presented for the heat-inactivated preparation in the presence of the different salts

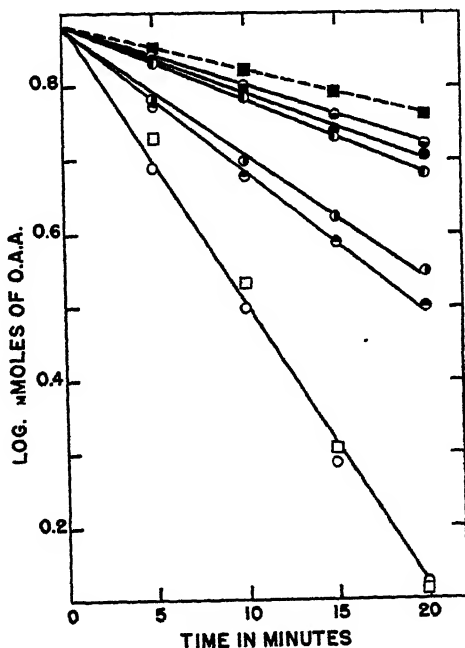


FIG. 2. Inhibition of the enzyme by salts giving only monovalent ions in solution. One-half ml. of 0.2 *M* acetate buffer, 0.5 ml. of salt solution; final ionic strength after dilution 0.012; 1 ml. of enzyme; 1 mg. of oxalacetic acid (O.A.A.) in 0.1 ml.; water to make 3 ml.; pH 4.0. ○ Control; ■ heat-inactivated enzyme. Salts used: □ NaF, ● NaCl, ● NH<sub>4</sub>Cl, ● NaClO<sub>4</sub>, ● NaNO<sub>3</sub>, ● NH<sub>4</sub>NO<sub>3</sub>.

because the variation in values was not greater than the variation in heat-inactivated control runs.

It will be noted that magnesium ion was used as a cation in these experiments. Speck (7) has shown that magnesium ion in itself will catalyze the decarboxylation of oxalacetic acid. Vennesland (3) has reported that metal ions do not, however, activate crystalline cucurbit

seed globulins. In the concentration at which magnesium was employed in these experiments it caused no measurable increase in the rate of decomposition of oxalacetic acid.

An attempt was made to observe whether the various ions influenced the metal-ion-catalyzed decarboxylation of oxalacetic acid in acetate buffer at pH 4. For these measurements cupric acetate giving a

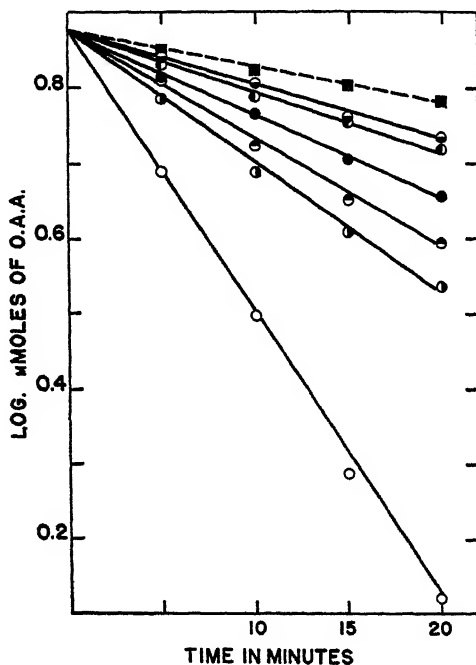


FIG. 3. Inhibition of the enzyme by salts giving divalent anions in solution. One-half ml. of 0.2 *M* acetate buffer, 0.5 ml. of salt solution; final ionic strength after dilution 0.012; 1 ml. of enzyme; 1 mg. of oxalacetic acid (O.A.A.) in 0.1 ml.; water to make 3 ml.; pH 4.0. ○ Control; ■ heat-inactivated enzyme. Salts used: ● Na oxalate, ● MgSO<sub>4</sub>, ○ (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, ○ Na<sub>2</sub>SO<sub>4</sub>.

final molarity of 0.0005 and manganous chloride of 0.01 final molarity were employed as catalysts. Keeping the ionic strength constant, substitution of acetate buffer with sodium nitrate and sodium perchlorate, in concentrations which had inhibited the enzyme reaction by 90%, gave no measurable change in rate of the reaction catalyzed by the metal ions.

Considering the results with mono- and divalent anions as a whole, it will be noted that those salts having a common cation, but different anion, such as sodium fluoride, sodium chloride, and sodium nitrate gave systems of considerably different reactivity, whereas salts having a common anion such as sodium nitrate and ammonium nitrate, or two similar salts of sulfate, gave systems of similar reactivity. Therefore, it may be concluded that at constant ionic strength, if no polyvalent cation activation is present, the activity of the enzyme is dependent on the nature of the anion or anions present. In order, then, to explain the different activities when the different salts were added, it appeared necessary to postulate that essentially two effects were being exerted by these ions on the enzyme system. The first was an inhibition dependent upon the ionic strength of the various components of the system. The second was a general base catalysis dependent upon the basic strength of the anion employed either in the buffer or as the added salt.

Such a hypothesis can be made by assuming that monovalent salts of nitrate, sulfate, and perchlorate are not reactive, but contribute to the system only in so far as they contribute to the ionic strength. This assumption has been substantiated in numerous other experiments, as indicated by Hammett (12). On the other hand, monovalent salts of fluoride, citrate, and acetate, the anions of which are good bases, in addition to contributing to the ionic strength, might actually enter into the reaction. Thus, when a portion of the acetate, a strong base, was replaced by nitrate, which contributes only to the ionic strength, the activity of the system decreased (as shown in Fig. 2). Whereas, when acetate buffer was replaced with fluoride, which is also a strong base, the activity of the system was essentially unchanged (also shown in Fig 2.)

It was, therefore, desirable to perform experiments which would allow a separation of the individual contributions of the basic, anion catalysis, and of the ionic strength inhibition. Further studies were undertaken using acetate buffer as a strong basic catalyst and sodium nitrate and sodium sulfate as nonreacting salts which contribute only to ionic strength. In Fig. 4 are presented data for experiments in which the ionic strength was kept constant and the concentration of acetate, the base, was varied. The ionic strength in this case was maintained at 0.0062 by adding varying quantities of sodium nitrate. This ionic strength was chosen because at higher acetate concentrations the ionic strength of the acetate itself materially inhibited the reaction. Activity,

in this and in the subsequent experiments, is expressed as the difference between first-order rate constants in the presence of heated and unheated enzyme preparation divided by the weight (in milligrams) of protein present. It will be noted in Fig. 4 that at constant ionic strength the activity of the system is directly proportional to the concentration of the acetate. The method of least squares was used in this and the subsequent experiments to fit the best straight line to the experimental points.

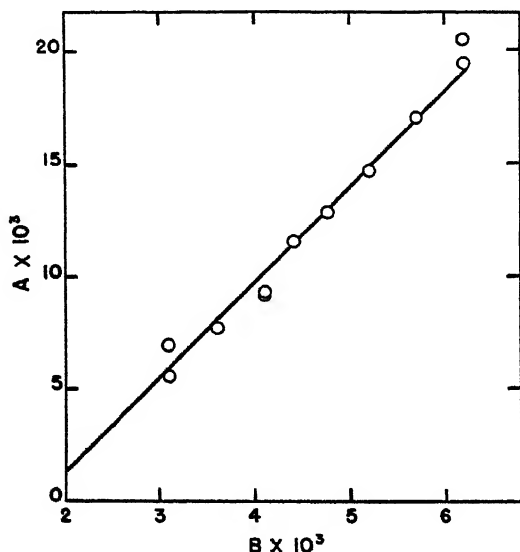


FIG. 4. Activity of oxalacetic decarboxylase in solutions of varying acetate concentration at constant ionic strength. Ordinate is activity ( $A$ ) expressed as the difference between the first-order rate constant in the presence of unheated and boiled enzyme, divided by the weight of protein in milligrams. Abscissa is acetate molarity ( $B$ ). Final ionic strength 0.0062; final volume in flask 3 ml.; pH 4.0; 1 mg. of oxalacetic acid.

In Fig. 5 are presented data for experiments in which the acetate concentration was maintained constant but the ionic strength was varied. Variations in ionic strength were accomplished by changing the concentrations of sodium nitrate or sodium sulfate. The concentration of acetate in all cases was 0.0052  $M$ . The data are plotted as logarithm of activity vs. the logarithm of the ionic strength. A straight-line relationship exists and the formula for this straight line may be represented

as follows:

$$\log A = (-3 \pm .2) \log \mu + \log k,$$

where  $A$  is activity,  $\mu$  is ionic strength,  $k$  is the proportionality constant, and  $-3 \pm .2$  is the observed slope. If the equation is expressed in the exponential form it may be seen that the following is obtained:

$$A = k \frac{1}{\mu^3}.$$

It is, therefore, indicated that at constant acetate concentration, activity is proportional to the reciprocal of the cube of the ionic strength.

To ascertain the independent influence of the basic anion catalysis and ionic strength inhibition in another way, an experiment was per-

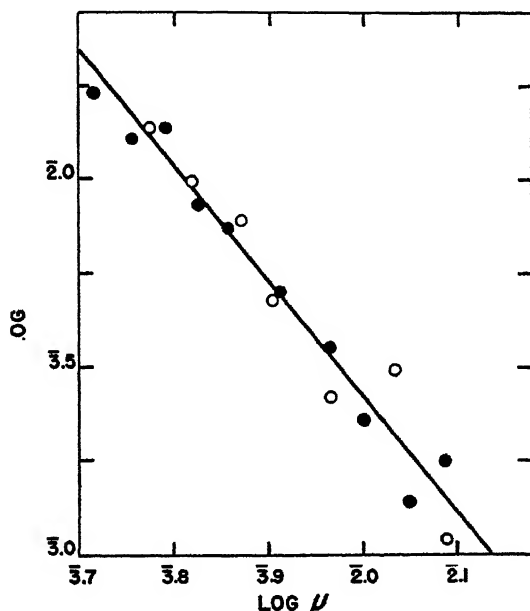


FIG. 5. Activity of oxalacetic decarboxylase in solutions of varying ionic strength at constant acetate concentration. Ordinate is log activity ( $A$ ), expressed as the difference between the first-order rate constant in the presence of unheated and boiled enzyme, divided by the weight of protein in milligrams. Abscissa is log ionic strength ( $\mu$ ). Final base concentration 0.0052  $M$ ; final volume in flask 3 ml.; pH 4.0; 1 mg. of oxalacetic acid; ●  $\text{NaNO}_3$ ; ○  $\text{Na}_2\text{SO}_4$ .

formed in which both ionic strength and acetate were varied by changing the concentrations of acetate buffer used in the system. No other ions were added. The data are presented in Fig. 6. It will be noted that a straight-line plot is obtained if it is assumed that the activity of the system is proportional to the acetate concentration and inversely

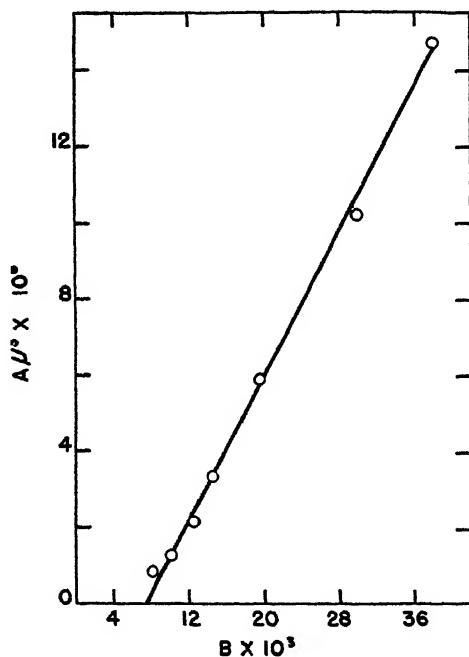


FIG. 6. Activity of oxalacetic decarboxylase in solutions of varying acetate concentration and ionic strength. Ordinate is activity ( $A$ ), expressed as the difference in first-order rate constants in the presence of unheated and boiled enzyme, divided by the weight of protein in milligrams times the cube of the ionic strength ( $\mu$ ); abscissa is molarity of acetate ( $B$ ). Final volume in flask 3 ml.; pH 4.0; 1 mg. of oxalacetic acid.

proportional to the cube of the ionic strength, in agreement with the data presented in Figs. 4 and 5. The concentration of acetate was changed in this experiment from 0.0084  $M$ , which approached the lowest concentration of buffer at which the pH could be maintained, to 0.038  $M$ , a concentration at which the effect of the ionic strength had almost completely inhibited the enzyme system.

*Effect of Biotin on the System*

Inasmuch as some evidence has been presented (13, 14, 15) that biotin possesses a coenzyme function, it was thought advisable to investigate the possible existence of a biotin-protein enzyme as well as a metal-protein enzyme. The natural biotin content of several globulin samples was determined microbiologically, but no correlation could be found between the biotin content and the activity of the enzyme. Efforts to affect the rate of carbon dioxide evolution by adding biotin to the enzyme system were also unsuccessful. A large excess of biotin<sup>3</sup> (20 µg./flask) had no measurable effect on the activity. The above results are similar to those obtained by Vennesland *et al.* (1) on the parsley root enzyme.

It was thought possible that removal of the biotin already present in the globulin might result in the lowering of the enzyme activity. To accomplish the removal, use was made of the egg-white protein avidin<sup>4</sup> which has been shown to combine quantitatively with biotin *in vitro* (16). Avidin was added to the system in an amount at least 370 times as great as was necessary to combine with all the biotin present, on the basis of the microbiological assay. After contact for 0.5 hr. at room temperature the activity was found to be uninhibited when compared with the control enzyme. It was concluded that a biotin-protein oxalacetic decarboxylase was not present in our preparation.

## DISCUSSION

From the results of the foregoing experiments, the activity of oxalacetic decarboxylase prepared, as the crystalline globulin, from cucurbit seeds may be expressed as follows:

$$A = K \frac{B}{\mu^3}$$

where  $A$  is the activity expressed as the difference between the first-order rate constant for the decarboxylation of oxalacetic acid in the presence of the active enzyme and the first-order rate constant for the heat-inactivated enzyme preparation, divided by the weight (in milligrams) of protein used.  $B$  is the concentration of acetate in the

<sup>3</sup> The sample of biotin used was obtained from General Biochemicals, Inc. in crystalline form.

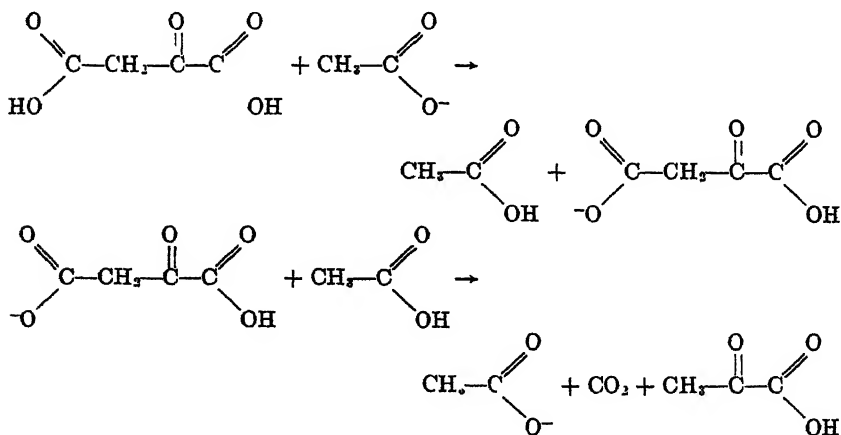
<sup>4</sup> A sample of avidin was obtained through the courtesy of Hoffmann-LaRoche, Inc.



acetic acid-acetate buffer employed, and  $\mu$  is the ionic strength.  $K$  is a constant which includes two proportionality constants. In all three experiments presented in Figs. 4, 5, and 6, which were performed under varying conditions of acetate concentration and ionic strength, the relationships expressed in the equation appear to hold. It is, therefore, indicated that the equation proposed above is general for the oxalactic decarboxylase system in which the only basic catalyst is the acetate buffer.

It may be noted, however, that if acetate were replaced with some other basic ion or buffer (as indicated in Figs. 2 and 3), different relationships might be obtained. One important aspect of these results is the indication that small changes in the concentration or basicity of the buffer, and of any other added ions, may have a large effect on the enzyme activity. It would, therefore, require care to select a test system from which the results could be related to physiological conditions.

A study of general acid-base catalysis of decarboxylations as reviewed by Hammett (12) indicates that in the decarboxylation of oxalacetic acid, the acetate may be catalyzing the reaction in the following manner:



This mechanism would explain the finding that the reaction is proportional to the first power of the acetate concentration.

It does not, of course, show or explain the effect of the enzyme itself, without which the basic catalysis is not ascertained, nor does it show the role which ionic strength plays in the reaction. As the ionic strength

increases, however, the effective concentration (activity) of acetate in the solution would decrease, and thus enzymatic activity would be less. Whereas the reduction in effective acetate concentration may be part of the ionic strength effect, the accompanying changes in the charge and hydration of the protein may also result in decreased enzyme activity. Therefore, because of the possible multiplicity of ionic strength effects, it is not possible to explain with certainty the finding that enzyme activity is inversely proportional to the cube of the ionic strength.

The presence of heavy metals, especially manganese, indicates that the enzyme may be a metal-protein complex similar in chemical characteristics to the exopeptidases studied by Smith (17). These findings may also explain the activator role of heavy metal ions on oxalacetic decarboxylase studied by other investigators (6, 7). It should be pointed out, however, that in an evaluation of the activation by different polyvalent cations, the effect of the accompanying anion must also be ascertained, especially if different anions are employed.

#### ACKNOWLEDGMENTS

The authors wish to express their thanks to Mr. H. L. Taylor of the Michigan Department of Health, Lansing, Michigan, for making the electrophoretic analysis, to Mr. H. V. Ogle of this department for his assistance in making the spectrographic determinations, and to Messrs. T. Chulski, O. Lien, and D. Waghorne for preparing the globulin samples used in this work. We would also like to thank Dr. J. C. Speck for his helpful criticism.

#### SUMMARY

1. A crystalline globulin preparation from *Cucurbita pepo* seed, which was observed to possess oxalacetic decarboxylase activity in direct proportion to the quantity of protein present, was essentially electrophoretically homogeneous under the conditions employed. An optimum pH for the reaction in acetate buffer was observed between 4.0 and 4.5.

2. Several heavy metals, principally manganese and iron, were found to be present in the ash of the purified protein.

3. No correlation was found between biotin content of the globulin and its enzymatic activity.

4. Independent of activation by polyvalent cations, two additional ion effects were noted to be influencing the enzyme system. The first was a general base catalysis found in these studies to be proportional

to the concentration of the base, acetate, present in the system. The second was an inhibition of the enzyme proportional to the cube of the ionic strength.

5. The significance of these findings with regard to effects of the buffering system and anion influence in polyvalent metal ion catalysis is discussed.

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## LETTERS TO THE EDITORS

### A Further Study of Pentolysis

In recent publications Menkès (1) has shown that the blood of cancer patients has the ability to destroy pentoses when added to it. For this purpose D(-)-ribose was used, but L(+)-arabinose or D(+)-xylose can be employed. The decrease of the sugar added indicates the *pentolysis*. Accordingly, a study on this pentolytic property was undertaken, using rats injected with a single subcutaneous dose of 20-methylcholanthrene (3 mg./0.5 ml. of mineral oil). Fifty-seven rats were injected in the abdomen, and blood samples were drawn by intracardiac punctures. Twenty-eight rats died before a blood sample was drawn, or showed no pentolysis<sup>1</sup> up to the time of their death (the latest death being 110 days after the injection). The first positive pentolysis (5% or greater) was noted within 79 days, and by the 219th day all the remaining rats were positive. A control group of 15 rats was injected in like manner with mineral oil only; four died prior to a blood sample being drawn, and no pentolysis (*i.e.*, less than 5%) was found in the remainder (the last blood sample being drawn 246 days after the injection).

It appears from the results obtained that the pentolytic property of the rats so-treated is not a late manifestation of the cancerization phenomenon.

Experiments are in progress to clarify the nature of this pentolysis, in particular as compared with glycolysis, and to shorten the chemical determination<sup>2</sup> of this pentolytic property so that a correlation between blood chemistry and pathology can be made. Additional experiments are planned with tissue slices and tissue cultures.

<sup>1</sup> Determinations of pentolysis were carried out by Dr. André Bopp.

<sup>2</sup> The remaining pentose after a 2-hr. incubation period is determined by a modification of the furfural method of Brachet (2).

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### The Metabolic Breakdown of Sarcosine by a Strain of *Pseudomonas aeruginosa*

In a previous communication (1), an atypical strain of *Pseudomonas aeruginosa*, characterized by its ability to decompose creatinine, was described. It was reported that cultures of this strain lost their viability within 8–10 days when grown in a 2% creatinine nutrient-agar medium. In this study, the same delayed lethal effect was obtained by cultivating the organism, preferably in shallow layers, in nutrient broth or *M/15* phosphate buffer of pH 7 to which creatinine, creatine, or sarcosine in a concentration of 0.13 *M* had been added.

Since the enzymatic decomposition of creatinine and creatine presumably yields sarcosine (2), our efforts were concentrated on the identification of breakdown products of the latter compound by the organism. By the use of chromotropic acid and dimethyldihydroxyresorcinol (dimedon), as described by MacFadyen (3), both free and bound formaldehyde were detected in all media in which the bacteria had died. Free formaldehyde could be removed quantitatively from the culture fluids with an excess of a 0.4% solution of dimedon in distilled water and identified by the melting point of the precipitated derivative (190°C.). The observed lethal effect is elicited only by free formaldehyde which accumulates in old, autolyzing cultures.

Resting cells of *Pseudomonas aeruginosa* failed to develop any formaldehyde from sarcosine; after treatment with toluene, however, the cells produced formaldehyde from the substrate. No formaldehyde was obtained when autolyzed cells were allowed to act at neutral pH on glycocyamidine, hydantoin, DL-alanine, glycine, acetic acid, glycolic acid, and glyoxylic acid. This led to the assumption that formaldehyde

was derived from sarcosine by oxidative demethylation in accordance with the following equation:



The presence of an enzyme catalyzing this reaction has been reported in mammalian livers by Handler, Bernheim, and Klein (4).

To determine the products of decomposition of sarcosine, the following experiments were set up. Amounts of 0.5 ml. of live or autolyzed organisms, corresponding to about  $5 \times 10^{10}$  cells, were added to 4.5 ml. of phosphate buffer of pH 7, containing the requisite amount of substrate, in test tubes of 1-in. diameter. The tubes were shaken vigorously in a 37°C. water bath for 3 hr. The bacteria were then removed by centrifugation and aliquots of the supernatants analyzed for formaldehyde, amino nitrogen, carbon dioxide, and volatile base. Formaldehyde was determined with chromotropic acid; nitrogen, developing in the presence of nitrous acid from amino groups, but not methylamino groups, was measured in a Koch-modified Van Slyke apparatus, carbon dioxide in a Van Slyke carbon dioxide apparatus, and volatile base by steam distillation into a flask containing hydrochloric acid and subsequent titration of the excess hydrochloric acid with sodium hydroxide. Identical determinations were carried out with controls of bacterial

TABLE I

*Action of a Strain of Pseudomonas aeruginosa on Sarcosine and Formaldehyde*

Type of cells	Substrate	Amount of substrate	Products of decomposition				
			Formaldehyde		Glycine <sup>a</sup>		Carbon dioxide
			Amount recovered	Per cent of theoretical yield	Amount recovered	Per cent of theoretical yield	Amount recovered
Autolyzed	Sarcosine	mg. 20.0	mg. 2.5	35.6	mg. 6.15	36.5	mg. 0
Resting	Sarcosine	20.0	0	0	12.25	72.7	0.2
Resting	Formaldehyde	0.3	0	0	— <sup>b</sup>	— <sup>b</sup>	— <sup>c</sup>

<sup>a</sup> In the absence of volatile base calculated directly from the amount of amino nitrogen found.

<sup>b</sup> Not determined.

<sup>c</sup> Amount found too small for evaluation.

cells in phosphate buffer without substrate. These were taken into account in the calculation of the final values. No volatile base was produced from sarcosine by either autolyzed or resting organisms. Other results are presented in Table I.

The secretion by the organism of an enzyme oxidizing sarcosine to formaldehyde and glycine seems confirmed. The fate of formaldehyde in the presence of live bacteria remains unknown. MacFadyen (3) incubated a suspension of *Shigella sonnei* with formaldehyde and subsequently succeeded in liberating up to 12% of the original amount in the course of the chromotropic acid reaction. His results could not be duplicated with centrifugates of resting cells of *Pseudomonas aeruginosa*. Only a very small amount of formaldehyde was apparently oxidized to carbon dioxide. The demonstrable absence of volatile base constitutes a correction of data published in a previous report (2).

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### Effect of Insulin on Adenosinetriphosphatase Activity<sup>1</sup>

In a recent note, Polis, Polis, Kerrigan, and Jedeiken demonstrated an effect of insulin on aerobic phosphorylation (1). They also described an inhibitory effect of the soluble magnesium adenosinetriphosphatase (ATPase) of muscle on this reaction and reported that insulin reversed this inhibition. Conceivably the antagonistic action of insulin and of the ATPase preparation could be due to one of several mechanisms. The ATPase could have acted as a result of its ability to split adenosinetriphosphate (ATP), and insulin could have inhibited this dephosphorylation. Or, the ATPase preparation could have acted on one or

<sup>1</sup> Aided in part by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

more of several of the phosphorylated intermediates other than ATP, either by virtue of a property inherent in the ATPase or by virtue of the activity of a contaminating system, and insulin could have inhibited a reaction other than dephosphorylation of ATP. In an attempt to circumscribe the action of insulin in this system, we have investigated the effect of insulin on a soluble ATPase preparation similar to that used by Polis and his colleagues (2).

TABLE I

*Effect of Insulin on Soluble Adenosinetriphosphatase*

Reaction mixture: 1 ml. 0.1 *M* histidine, pH 6.8; 0.5 ml. ATP (containing 300  $\mu$ g. P<sub>i</sub>); 0.1 ml. 0.15 *M* MgCl<sub>2</sub>; 0.2 ml. ATPase; insulin and H<sub>2</sub>O to 3 ml. total volume. Incubated 5 min. at 38°C. Reaction stopped with trichloroacetic acid; inorganic phosphate determined by the method of Lowry and Lopez (3).

Experiment	Insulin	ATPase	$\Delta P_i$
	<i>units</i>		<i><math>\mu</math>g.</i>
1	0	0	1.2
	0	+	51.5
	0.1	+	53.8
	1.0	+	53.8
2	0	+	32.3
	0.2	+	29.7
	0.4	+	31.0
	0.6	+	31.3
	2.0	+	33.2
3	0	+	11.0
	0.2	+	11.6
	0.5	+	10.7
	1.0	+	13.1
	2.5	+	12.0
	4.0	+	12.6

The effect of insulin was determined in ten trials on the ATPase activity of eight separate preparations of this enzyme system. Varying quantities of enzyme and insulin were tested. In these ten trials with preparations of varying activity, no evidences of a significant inhibition by insulin could be adduced. Representative results of these trials are listed in Table I.



From these data, it would appear that the mutually antagonistic effects of insulin and the soluble adenosinetriphosphatase preparation on the process of aerobic phosphorylation must be ascribed to some reaction other than the splitting of adenosinetriphosphate. It remains to be determined whether insulin affects another reaction catalyzed by the ATPase or a different reaction catalyzed by another enzyme system coincidentally, and not always present as a contaminant.

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## Book Reviews

**A Manual of the Penicillia.** By KENNETH B. RAPER, Principal Microbiologist, Fermentation Division, Northern Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture, Peoria, Illinois, and CHARLES THOM, Collaborator, U. S. Department of Agriculture, formerly Principal Mycologist, Bureau of Plant Industry, U. S. Department of Agriculture, Peoria, Illinois. The Williams & Wilkins Company, Baltimore, Md., 1949. ix + 875 pp. Price \$12.00.

This very welcome book makes available for the rapidly growing group of scientists who are interested in molds belonging to the genus *Penicillium* the ripe experience and profound knowledge gained by Dr. Charles Thom over a period of almost half a century. In the writing of this book he has been fortunate in having as joint author his former pupil, Dr. Kenneth B. Raper. Many of their British friends have been looking forward eagerly for some time to the appearance of the "Manual of the Penicillia," and it affords real pleasure to be able to state unreservedly that their hopes have been abundantly realized.

Thom began to study *Penicillia* in connexion with cheese investigations in 1904, published "Fungi in Cheese Ripening (Camembert and Roquefort)" in 1906, his "Cultural Studies of *Penicillium*" in 1910, and his group concept of classification of the *Penicillia* in 1915. In these papers the necessity of comparative culture upon standardized media was stressed in contrast to the search for optimum conditions of culture, organism by organism. Then in 1930 there appeared Thom's classical monograph, "The *Penicillia*." The authors of the present volume define the duties of a monographer as follows: "The first obligation of a monographer of the *Penicillia* is to report as truly as possible what his predecessors described under particular names. His ideal is to produce as complete and as faithful a presentation as possible of the work of his predecessors, supplemented by his own observations and knowledge." With the passage of years Thom's outlook has to some extent changed, and in the "Manual of the *Penicillia*," based on a study of more than four thousand strains, the authors have dropped the monographic feature of Thom's 1930 publication. In the Manual the authors define their duty as follows: "In contrast to the monographer, the writer of a manual of the *Penicillia* must begin with the establishment of a genus concept, then account for all species that have been assigned to it. He may correct, re-describe, reassign, or reduce to synonymy any specific name and description encountered, so long as his own descriptions lead to the identification of actual material, and to the assignment to that material of Latin names which are correct according to accepted rules of nomenclature. His primary obligation is to the investigator who needs to identify an organism." This duty they have abundantly discharged.

The Manual is divided into three parts, and each part into a number of chapters.

### Part I. General Discussion

The five chapters in this part covering 104 pages are headed: Historical; Generic Diagnosis and Synonymy; Observation and Description of Penicillia; Cultivation and Preservation of Penicillia; Penicillin. Biochemists will find the chapter on penicillin a very well informed article. In the short space of sixteen pages it gives an authoritative account covering almost every aspect of the subject, and for those who wish more detailed information there is an excellent bibliography later in the book.

### Part II. The Manual Proper

The ten chapters in this part, which covers 568 pages, are headed: Use of the Manual; Monoverticillata; Asymmetrica-Divaricata; Asymmetrica-Velutina; Asymmetrica-Lanata; Asymmetrica-Funiculosa; Asymmetrica-Fasciculata; Biverticillata-Symmetrica; Polyverticillata; Gliocladium, Paecilomyces, and Scopulariopsis.

It is of interest to compare this part of the book with Thom's "The Penicillia" (1930). The classification of species follows, in general, the scheme laid down by Thom in 1930. The criterion for the first main division of the genus is the type of branching of the fruiting organs (penicilli). There are thus four main sections, Monoverticillata, Asymmetrica, Biverticillata-Symmetrica, and Polyverticillata. In each of the first three main sections, first consideration is given to species which produce perithecia or sclerotia. Since 1930 a fairly large number of ascosporic species have been described. Since such species are by no means so uncommon as was at one time believed, it is very useful to have the material from many scattered publications gathered together and critically evaluated. The great majority of species, which produce neither perithecia nor sclerotia, are further classified mainly on the basis of colony texture. The soundness of Thom's taxonomic work (1930) is indicated by the fact that the majority of new species described since 1930 are accepted as valid in the present manual.

The first of the keys, p. 120, is a key to series only and is in diagrammatic form. This is what anyone who is trying to identify a *Penicillium* will turn to first, and the clear way in which it is laid out deserves nothing but praise. A useful feature of the keys to the various subsections and series is that they are well provided with cross references to other groups. This makes it possible to place abnormal strains, such as, for example, color mutants. My mycological colleague, Mr. George Smith, is, however, of the opinion that it is a pity that the dichotomous system was not used for all the main keys, since he believes that this system is much more satisfactory than the one adopted in the present manual.

In spite of the description since 1930 of a fair number of new and good species, the total number of species accepted in the Manual is much less than the number accepted by Thom in 1930. In his 1930 monograph Thom recognized that some ubiquitous species show a certain amount of natural variation, and he introduced the conception of the "group species." This conception has now been extended to cover most of the common species, including a number which had been inadequately investigated up to 1930.

A few species have been transferred to quite different series, e.g., *Penicillium puberulum*, *P. aurantio-virens*, *P. aurantio-griseum* var. *poznaniensis*, and *P. griseo-fulvum*. It is interesting to note that the new placings are confirmed by the biochemical characteristics of the species concerned.

Comparatively little is added to Thom's 1930 discussions of the related genera,

*Paecilomyces* and *Scopulariopsis*. *Gliocladium* is somewhat better served. In view of the enormous amount of work which has been required to straighten out *Penicillium* proper it was perhaps too much to expect that *Paecilomyces* and *Scopulariopsis* should receive adequate treatment. Nevertheless, a thorough and detailed study of these genera of common molds is urgently required.

All biochemists, including those with some knowledge of mycological chemistry, will find the paragraphs entitled "Occurrence and Significance" exceedingly valuable since in them is summarized almost all the available information on the physiology, biochemistry, and pathogenicity of species of *Penicillium*. Further, in those cases where the authors have been unable to trace any such information they say so quite clearly. There is a quite extensive account of mold metabolic products, which are so surprising in their numbers and almost bewildering in their chemical structures. In addition there is a very extensive bibliography to which reference will be made later.

### *Part III. Reference Material*

The four chapters in this part, which covers 146 pages, are headed: Topical Bibliography; General Bibliography; Species Index; Accepted Species and Varieties.

In the Topical Bibliography covering 46 pages, the references to papers, of which all titles are commendably given in full, are arranged under a variety of subject headings including, for example, acid production, antibiotics, cheese, deterioration and spoilage, enzymes, fat production, metabolic products, pathogenicity, penicillin, pigments, and polysaccharides. In the General Bibliography of 43 pages, the titles of the papers quoted are also given in full and are arranged under authors' names which are given in alphabetical order. Many of the references given in the Topical Bibliography are repeated in the General Bibliography, but this is not a serious fault since it often enables one to track down a reference more easily. The Check List of Species and Genera consists of two parts. The first part (2 pages) gives a list of all those generic names which have been found applied to *Penicillia*. The second part (48 pages) gives a Check List of all named species of *Penicillia* together with literature references, and in those cases, surprisingly large in number, where the previously assigned species names are not accepted by Raper and Thom, they suggest probable synonyms. Finally there is a list of 142 accepted species and varieties together with authorities.

The Manual closes with an adequate general index.

No review of this Manual would be complete without reference to the 172 photographs and line drawings, the work of Miss Dorothy I. Fennell and her collaborators. They are a very useful feature of the book and with very few exceptions are well executed. There is a particularly beautiful and characteristic photograph of *P. claviforme* on page 552. There are also ten natural color photographs of different species of *Penicillium*. A list of illustrations with page numbers would have been useful and it would add to the usefulness of the reference to Color Photographs of *Penicillium* given in the general index if the page numbers were cited in future editions.

The general format of the Manual is good and the printing and paper are excellent. Misprints are refreshingly few and are mostly obvious. The reviewer has however noted the following errors. On page 315 reference is made to two strains of *P. ochrochloron*, isolated by Sato, which are strikingly resistant, the one to copper sulfate and the other to sulfuric acid. There is considerable confusion in factual statements made

about these two strains. On page 418 it is stated that "Abraham (1945) reported culture liquors from *P. brevis-compactum* to contain normycophenolic acid ( $C_{16}H_{18}O_6$ ). The compound was in fact mycophenolic acid ( $C_{17}H_{20}O_6$ ). Finally the reviewer believes that the authors are in error in describing the origin of Sopp's original strain of *P. islandicum*. In Thom's 1930 monograph, p. 466, it is stated "species found on the island of Skyr and a variety upon Stereum in Norway." In the Manual, p. 625, the authors state "Sopp based his species upon a culture found on the Island of Skyr, Norway." Personal enquires by the reviewer at the Icelandic Legation in London elicited the fact that Skyr is a bacterially soured milk peculiar to Iceland and is somewhat similar in nature to Bulgarian yogurt. The error seems to have arisen from a mistranslation of Sopp's original description which states "der Pilz wurde auf isländischem Skyr gefunden," and Sopp himself states quite definitely that he isolated the mold in Norway from a moldy specimen of Skyr received from Reykjavik, Iceland.

The reviewer recommends the Manual to all biochemists and others who are interested in molds in the sure and certain belief that it is and will remain for a number of years the finest publication on the genus *Penicillium* in any language.

HAROLD RAISTRICK, London, England

**Advances in Food Research.** Vol. II. Edited by E. M. MRAK and GEORGE F. STEWART. Academic Press Inc., New York, N. Y., 1949. xiii + 558 pp. Price \$8.80.

During a period, the end of which is not yet in sight, when an ever increasing mass of scientific literature has made impossible the attainment of a satisfactory background in more than one isolated field of endeavor, several series of review volumes have appeared. Although these symbols of man's inadequacy to cope with an environment that he has created for himself have also become numerous, they do represent a partial solution to a difficult problem. The usefulness and success of these volumes are directly related to the effort and thought applied by and the degree of cooperation among the publisher in his presentation of material submitted, the editor in his selection of subjects for review, and the authors in their correlation and assessment of subject matter.

Volume II of the series "Advances in Food Research" is an excellent example of what can be accomplished by intensive effort on the part of all members of a review group.

The publishers have maintained the standards established for the first volume. The general presentation of material is excellent, the type is easy to read over relatively long periods of time, the tables, cuts, and figures are remarkably clear and understandable, and the indexes are adequate.

The editors are to be commended for the selection of subjects that will find wide and immediate interest among food technologists and others associated with the food industry. Those not closely allied to the food industry will also discover much that will enrich and perhaps justify research in fundamental specialties. Four of the reviews cover important phases of processing, three are related to bacteriological applications, five comprise or include discussions concerning chemistry, three deal with food flavors. Other fields encompassed, in part at least, include pharmacology, toxicology, and general methodology. For those who are not already familiar with the scope of this volume, the subjects reviewed are: Ion-Exchange Application by the

Food Industry; Thermobacteriology as Applied to Food Processing; the Quarternary Ammonium Compounds and Their Uses in the Food Industry; the Pharmacology of DDT; Analysis of Foods by Sensory Difference Tests; the Chemistry of Fruit and Vegetable Flavors; Histological Changes Induced in Fruits and Vegetables by Processing; the Spoilage of Fish and Its Preservation by Chilling; and Spray Drying of Foods. All of the reviews are pertinent to present-day problems with which the food industry is faced.

The authors in general have given ample evidence of their abilities to discover, analyze, assess, and interpret the mass of material that has accumulated in their respective fields during recent years. Literature references are abundant and should prove extremely useful not only to active workers as means for checking their own specific knowledge and coverage but also to others who would like to locate other recent reviews and major publications as a basis for extending their general knowledge.

This writer has been particularly pleased with the presentations on the chemistry of fruit and vegetable flavors, on the chemical and flavor changes in fish, and on the analysis of foods by sensory difference tests. This last review will have great value to those interested in the quality and acceptance of food products, inasmuch as there is no other similar discussion elsewhere in the literature.

Although continued excellence of this series seems assured, it may be hoped that Volume III will follow Volume II more rapidly than the latter followed Volume I.

E. E. LOCKHART, Cambridge, Massachusetts

**The Basis of Chemotherapy.** By THOMAS S. WORK, Research Staff, National Institute for Medical Research, London, and ELIZABETH WORK, Research Staff, Department of Chemical Pathology, University College Hospital Medical School, London. Interscience Publishers, Inc., New York, N. Y., 1948. xx + 435 pp. Price \$6.50.

This is a concise and clear presentation of a difficult subject. The material has been selected with care and correlated with expert judgment. The authors are to be congratulated for the excellent way they have treated the complex data.

The introduction deals with the historical development of the facts leading up to the present knowledge of the important events in cell metabolism and their significance. The remainder of the book treats the subjects of cell metabolism, essential metabolites, enzyme inhibition, drug antagonism, drug resistance, and the relation of structure and activity. In every chapter, the facts are presented in an interesting manner and they are evaluated with fairness and precision.

The importance of enzymes in determining and regulating the cycle of events on which the life and activity of the cell depend is emphasized throughout and the nature and functions of the known enzymes presented cogently and tersely. It is shown that changes in enzyme reactions may have far-reaching consequences, reflected in altered cell functions and behavior of the organism. Survival of the cell may require a general readjustment of enzymatic balance resulting, not only in changes in the normal processes, but, likewise in altered food requirements, change in rate of growth and in resistance to drugs.

It is pointed out that chemotherapy is concerned with the destruction of the invading organism by drugs with least harm to the host. To be effective, drugs must reach

the site of the difficulty in unchanged form. There are several biological hurdles to overcome. The structure of the drug may be right, but the physical properties may prevent the product getting by the biological barrier. Much more must be known about the transport to and metabolism of drugs in biological systems. To know more of the mode of action of drugs, it is necessary to get a better understanding of the nature of all the important enzymes in both the cells of the microorganism and the host. Little is yet known about the enzymes involved in protein syntheses and degradation. Drugs or enzyme inhibitors may function by affecting the capacity of enzymes in their synthetic or their degradational reactions. With a thorough knowledge of the different enzymes and their properties, it may be possible to find the proper drugs for blocking specific and essential enzymatic reactions in the microorganism without too seriously affecting the cells of the host.

The book is a valuable digest of the available data concerning the basis of chemotherapy and will be, I am sure, a great aid to those interested in the study of the subject.

M. L. CROSSLEY, Bound Brook, N. J.

**Oxidation-Reduction Potentials in Bacteriology and Biochemistry.** 5th ed. London County Council, 1948, 130 pp. Price 4/6d.

This book, in its fifth revised edition, will amply serve to supply biologists, especially bacteriologists, with proper information of all attempts to utilize the concepts and methods concerned with oxidation-reduction potentials for the study of living systems, especially of bacterial cultures. The reader will learn very soon that the application of the theory of redox potentials as obtained in inanimate systems at equilibrium, to living systems is a precarious task. Although the reviewer appreciates the kind of presentation, yet he wonders whether the concept of "potential" has not been used too indiscriminately. Certainly, when a platinum electrode is immersed in a bacterial culture or in tissue extract, one can measure a certain potential, sometimes a well poised one. This potential, however, does not reflect the "potential" of the complex living system as a whole. Such a system contains a mixture of many redox systems, such as  $O_2 \rightleftharpoons H_2O$ ;  $O_2 \rightleftharpoons H_2O_2$ ;  $H_2O_2 \rightleftharpoons H_2O$ ; flavin dyes  $\rightleftharpoons$  their leuco dyes; coenzyme  $\rightleftharpoons$  reduced coenzyme; cytochromes, *etc.*, and possibly artificially added methylene blue and its leuco dye. Each of these systems is, in the steady state, partially reduced; and the ratio of each oxidized form to its corresponding reduced form may be said to reflect a "partial redox potential." If there were equilibrium, all partial redox potentials would be alike. In the living system, in the presence of oxygen (or, in the case of anaerobic organisms, some other ultimate oxidant, such as nitrate), the various partial potentials cannot be alike, as can be best seen from the fact that, *e. g.*, the  $O_2 \rightleftharpoons H_2O$  potential cannot be the potential measured at the electrode. One may say that the electrode does not "respond" so readily to the  $O_2$  potential as to the other partial systems. However, the electrode does not respond either to the succinate-fumarate potential even in the presence of succinic dehydrogenase. This enzymatic system reacts with methylene blue (or with the flavin dye) which, in its turn, will impress its partial potential at the electrode. The interaction of the enzyme-system with the dye is, however, a slow process, and, in the living system, the partial potential of the succinate system will always lag behind that of the dye system. What the elec-

trode measures is essentially only the partial potential of the dye system. It depends on the ratio of dye to leuco dye in the steady state. This ratio, in its turn, depends on the rate at which the dye is enzymatically reduced by combustible substrates, and the rate at which the leuco dye is oxidized by  $O_2$ , directly or indirectly, through the chain of enzymes. Consequently, the potential which can be measured at the electrode or by a redox indicator is just the partial potential of a system which is readily and spontaneously reversible, without the aid of an enzyme, such as the partial potential of an artificially added redox indicator, or the naturally occurring alloxazine dyes. Such dyes can also act as catalysts and so influence the "potential" as measured; a fact of which the author is well aware.

It is not implied herein that the author does not realize all these difficulties; and yet it seems that the reader after all may obtain a wrong impression of what those "potentials" mean, and fail to recognize that there is not such a thing as a redox potential in a living system.

However, these objections are by no means intended to discourage the methods used. They have very well served their purpose for characterizing various bacterial species and tissues. In whatever way the meaning of these potentials may be explained they are characteristic properties of the systems. This book gives a good account of all the material and, as it appears, a full report on the literature. It is to be recommended especially to the bacteriologist.

L. MICHAELIS, New York, N. Y.

**Experimental Cell Research.** Supplement 1. Proceedings of the 6th International Congress of Experimental Cytology. Edited by T. CASPERSSON and L. MONNÉ. Albert Bonnier, Stockholm. Academic Press Inc., New York, N. Y., 1949. xi + 603 pp. Price \$9.00.

Some twenty years ago it would have been easy to point to a journal or book dealing adequately with the subject of cell research. The favored conception of the cell as a colloidal framework had then been in the fullness of its development. Osmotic and swelling pressure, viscosity—structural and Newtonian, permeability, surface tension, and the manifold effects of adsorptive behavior, were the familiar criteria of reference. If cell physiologists as a group paid but a perfunctory deference to the striking developments in protein chemistry, metabolism, and cytogenetics, it was a limitation born not out of ignorance but out of the historical triumph which their studies had produced. Vitalism drew its last strong breath under the endless impacts of demonstrations that protoplasmic behavior could be mechanistically explained. Years after, faith in the sturdiness of this physico chemical view still found its echo in a journal like *Protoplasma*, and in many texts such as those of Höber, Scarth, Seifriz, and Heilbrunn. Few teachers of the subject questioned the adequacy of topical treatment; and probably fewer still imagined that progress in cell research might move in utterly new paths. The cell as a colloidal complex was, at least up to a point, an all-embracing conception.

Today, not even the most casual survey of cellular studies could fail to see the extreme departures in method and outlook which now dominate the field. Yet, however apparent these departures and however significant the contributions, the formal definition of the business of cell research has been hard in coming. The mechanistic



soundness of the classical conception favored extrapolation to innovation, while the long revered myth of the cell as *the* fundamental unit of research inclined more to preying upon allied fields of physico chemical studies for additional textual material than to the acceptance of the new if less sophisticated views. Recent attempts to revise cell physiology in terms of topics ranging from the physical process of diffusion to the biochemistry of redox systems have served only to underline the truism that the cell is the seat of most all biochemical and biophysical considerations. They have failed, though, to press home the point that the approach has shifted from a general description of physical and chemical properties to a more specific analysis of cell parts and their interrelations. The basis of contemporary cell research is the chemical analysis and the physical definition of identifiable cell structures. However tedious the approach may seem, it is a factor indispensable to the attempts now current in resolving the internal relations of cell behavior.

In the absence of a gentle transition from old to new, the rewriting of texts and the revision of journals have been difficult to achieve. Yet, so obvious has been the gap that the printing of this volume is an expected surprise. It makes its debut with much modesty being but a supplement to the forthcoming journal, *Experimental Cell Research*, and consisting simply of the papers presented at the Sixth International Congress of Experimental Cytology. It is thus in no sense a text, nor even a much needed review volume. Its appearance, nevertheless, is to be welcomed for it sets in motion the means whereby a more integrated and better discussed conception of the cell can be achieved. The contents, to be sure, will prove of variable interest according to the reader's predilections, but the broadness of subject matter provides ample cause for reflection on contemporary trends. The range of topics is well illustrated by the sectional titles: Chemical Constituents of Protoplasm; Submicroscopic Structure of the Cell; Nucleus, Chromosomes, Genes, and Mutations; Virus and Bacteria; Antigens and Serological Reactions; Muscle Chemistry and Structure; Cell Metabolism; Cell Permeability; Correlations between Structure and Function of the Cell; Tissue Culture; Developmental Physiology. In the face of so extensive a survey consisting of a hundred odd contributions, the reviewer must content himself with a few critical and somewhat general reflections.

Apart from those researches not dealing directly with the cell pattern, the studies reported fall largely into one of three categories:

- (1) Chemical composition of microscopically visible cell structures and structure of microscopic and submicroscopic particles.

- (2) The biochemical relations between such structures.

- (3) The sequence of physical and chemical events in the course of cell development. The first two are essentially considerations of spatial relations; the third, of time.

1. The one distinctive if unspectacular innovation in cell research has been the substitution of purely morphological designations by accurate chemical analysis. The benefits of histochemical and cellular fragmentation techniques are apparent in the majority of papers reported. If one adds to these the newer technique of electron microscopy—a subject dealt with by a number of contributors—it may be safely concluded that few studies of the cell can profitably ignore all of the three approaches. Admittedly, the methods are still crude and will bear much refinement, but the principle of chemical and physical identification of cell structures is firmly established.

It is as evident in the considered treatment of the bacterial membrane by Dubos (p. 192) as in the polemically concluded discussion of lipoproteins by Chargaff (p. 24). Even the once monarchical but woolly frame of reference—protoplasmic viscosity—has abdicated its post to the extent of being exclusively treated in 2 papers by Stålfelt (p. 63) and Virgin (p. 79). So too with permeability, except here one feels that the 22 pages devoted to it are in the nature of a halt, not an abandon. The remarkableness of selective permeability has lost little of its excitement; if many of its followers have been diverted, it is because a familiarity with the chemical composition of cell structures is at this stage the more fruitful venture. One outstanding effect of this methodical approach has been a discouragement of the irresistible speculation on the cell as a whole. And rightly so. The high complexity of its structure merits more than a passing glance into its chemical nature. Of the latter our knowledge is still meager, and the all-embracing hypotheses one occasionally meets may at best be regarded as curiously interesting. In such category one might place Bonnevie's treatment "On the Mechanics of Mitosis" (p. 100), and Bab's more imaginative project on "The Mechanism of the Metabolism Regulation in Cells" (p. 334). A fit comment on the conception that in mitosis "Two groups of autonomically working processes appear to cooperate intimately with the living cell, thus bringing about a typical mitotic cell division:" is the author's own—" . . . it [the hypothesis] has not yet met with any argument that would justify its rejection." Naturally; a generalization flushed with vagueness is too ethereal to mingle with mundane fact, and the experimentalist will dignify a speculation as hypothesis only when it invites a critical test of the facts. Bab's deification of the centriole as not only directing "the entire cell metabolism both chemically and physically during the vegetative stage" but also as being "the motor and regulatory force of the mitotic process" writes its own comment. Interestingly, the views of Bonnevie and Bab respecting the mitotic process, though divergent, are both equally immune to arguments that would justify rejection. The impression is strong that the ascendancy in use of analytical cell chemistry is largely removing the area of cell physiology from quasi-philosophical speculations.

2. If developments in microchemistry and optics have supplied the wherewithal for progress in cell analysis, it is cytogenetics which is responsible for initiating studies on the roles and interactions of cell organelles. The revision of cell physiology has been imposed from without, leaving the reciprocity of mild contempt between cytologist and cell physiologist as a faded mark of historical adolescence. Specifically, cytogeneticists forced the conclusion respecting the critical role of the nucleus in cell behavior and thus injected the fundamental and distinctly practical notion in experimental cell research that realistic treatment of the cell can be achieved only in recognition of the special influence of the nucleus as site of genetic factors. This viewpoint has now established itself, as perusal of the volume will show, in most fields of investigation—from reproductive tissues to virus and bacteria. The benefits of this conception have, furthermore, so overflowed that attention is being increasingly focused on the characterization of other organized localities of protoplasm by distinctive metabolic functions. Methods of cell analysis thus become a commonplace procedure, and the bolder role is assumed by investigation of the interaction between entities such as chromosomes, mitochondria, microsomes, viral complexes, nucleoplasm, and cytoplasm. It is understandable that conjectures on these relations are still in their infancy. The commonly cited correlations between nucleic acid concentrations and

synthetic activity, strongly emphasized, for example, by Koller (p. 85) and Serra (p. 111) have given birth to an inordinate amount of research on the role of nucleoproteins in cell behavior. So much so, that a correlation has even been sought between psychic functions and nucleoprotein metabolism (Hydén, p. 109). It is nevertheless possible that the rush by students of the cell to elucidate nuclear-cytoplasmic relations in terms of ribonucleic acid (RNA) and desoxypentose nucleic acid (DNA) has missed its mark by overrunning. The correlation, to be sure, has been frequently demonstrated but the mechanism of connection between the elaboration of nucleic acids and the synthesis of proteins is purely conjectural. The protein moiety of the chromosome complex has received much less attention than it deserves. The evidence, as presented by Serra, on the variable chromaticity of the chromosomes suggests much caution in the exclusive treatment of any single component. Moreover, taken at a cruder level, there is little factual data on the localities of specific syntheses. The statement by Weiss (p. 479) that "protoplasm seems to be synthesized exclusively at, if not inside, the nucleus" is more imaginative than daring, although his technique of constricting nerve fibers is elegant in approach and simple in method. The word "at" is a loose designation becoming looser still when transposed from neurones, upon which his conclusions are based, to cells of utterly different morphology and function. Moreover, synthesis of "protoplasm" has a most indefinite meaning. There is good reason not to suppose that all components of protoplasm are, with respect to locale, synthesized in identical manner. There is also compelling evidence leading one to regard as meaningful only those studies which specify both a specific synthesis and a particular phase of it. One would want to know which complexes are formed in the cytoplasm and which in the nucleus; which cytoplasmic materials are further modified in the nucleus and whether they are retained there or returned to the cytoplasm; and also, what substances elaborated in the nucleus act as directional influences in autonomous cytoplasmic syntheses. In this regard, Koller's article on "The Integration of Nuclear and Cytoplasmic Activities in the Cell" is instructive reading. It is regrettable that the field of work on particulate structures in cytoplasm is represented only briefly in a paper by Chargaff (p. 24). For developments here certainly emphasize a localization of metabolic activities as strong, though probably different in kind, as that found in the nucleus. The studies by Sheffield (p. 178) and Pirie (p. 183) or the formation of viral complexes with cytoplasmic components lend additional importance to the investigation of intracytoplasmic aggregates.

3. In comparing the section on developmental physiology with preceding parts of the book, the contrast in content and form is striking. It is hardly an issue of complexity of materials, for the respective complexities of the cell as a heterogeneous metabolic unit and as a developing entity are points too moot to question and pose an argument too provincial to be worth embracing. What begs reflection is the fact that the one, if unspectacular, is definitive in its approach, while the other, if vivid, is fuzzy in its experimental outlook. Two notable exceptions are the papers by Runnström on activation (p. 469) and by Monroy on the fertilization membrane (p. 525). For the rest, one feels as though enraptured by wandering minstrels of biology. There is little sufficiently compelling in any one of the experimental reports that could command an undivided following. Daleq's plea for the concept of "physiological competition" (p. 493) is as inviting as the report by Moricard and Gothie on "tensio-active substances" (p. 517). The longer one makes the list of views, the

stronger is the impression that embryology is still searching for an approach that is experimentally fruitful. It is interesting that the outstanding embryologist Holtfreter (p. 497) confines his remarks to the colloidal concepts—viscosity, sol-gel transformations, adhesion—now somewhat historic in cell research. Cytoplasmic division, for example, is ascribed to a constriction of the cell membrane, an explanation similar to that current in several old physiology texts. Beyond a shadow of doubt, any number of surface-active factors can produce changes in cell form, but specifically what does it in a given cell *in situ*? Nor is it helpful that “obviously in normal cell division there is a correlation between the process of mitosis and cleavage” for the mitosis of pollen cells proceeds without it, and one may thus add to Holtfreter’s list of cleavage with mitosis and cleavage without mitosis, mitosis without cleavage.

The smooth and marvellous functioning of the complex living organism inclines one impatiently—now, as always—to comprehensive explanations. Yet, however appealing universals may be, the need for a familiarity with details of structure and composition in the living cell is patent. It is in answer to this need that one recognizes the asset of the present volume and the journal it precedes. One might further add the hope that this book will be succeeded by ones of a more general review nature similar to the now familiar “Advances.”

HERBERT STERN, New York, N. Y.

**The Plant Alkaloids.** 4th Ed. By THOMAS ANDERSON HENBY, formerly Director, Wellcome Chemical Research Laboratories and Superintendent of Laboratories, Imperial Institute, London. The Blakiston Company, Philadelphia, Toronto, 1949. xxiii + 804 pp. Price \$14.00.

The appearance of the 4th ed. of this authoritative work after an interval of 10 years is most welcome to all those interested in the chemistry of natural products, and especially of alkaloids, which, after all, were, for centuries, among the most important of our medical and chemotherapeutic agents.

The interest and activity in the alkaloid field is reflected in the expansion of the present volume by some 115 pages beyond the third edition. In addition to the previously treated subjects, there are new chapters on the pyrrolizidine and steroid alkaloids, and a short section which lists by botanical name all plants in which the presence of alkaloids has been reported.

The introduction includes new material on the use of chromatography and ion exchange, and a brief discussion of important new developments in structure, and of synthetic substitutes for alkaloids in medicine.

Many sections covering groups where there has been little or no activity, such as the areca, pomegranate, coca, cactus, berberis alkaloids, and others of well-established structure, have been practically reprinted, with addition of a few new references. Others, such as the sections on cinchona, opium, strychnos alkaloids, and colchicine, have been largely rewritten to take account of the notable advances in these fields. Most of the chapters present a short report on pharmacology; that on the morphine group runs to 7.5 pages, which is perhaps not out of place in view of the importance of the drug and its substitutes. This results in a long-needed correlation of the extensive investigations on the chemistry and pharmacology of the morphine series carried out during the last 20 years. The difficult strychnine chapter is well written,

but could not, unfortunately, include the recent contribution of Woodward and Brehm on the anomalous behavior of methoxymethyl $\beta$ -cyanodihydrostrychnine, and its interpretation in terms of structure.

Each of the major sections has its own extensive and reliable bibliography. The literature of the 10-year period has obviously been followed with great care, and the references extend well into 1948.

This volume will serve as an indispensable source of information for chemists, biochemists, pharmacologists, and, to some extent, botanists, for many years.

LYNDON F. SMALL, Bethesda, Maryland

**Industrial Rheology and Rheological Structures.** By HENRY GREEN. John Wiley & Sons, Inc., New York, 1949. xii + 311 pp. Price \$5.50.

This is a very valuable and refreshingly unusual book. The author, who was a recognized leader in the science of rheology and microscopy, undertook the task to write a manual for the practical man working in an industrial laboratory on rheological problems. He incorporated in the book his life-long experience and succeeded admirably in creating a guide which provides the worker with the required minimum of theoretical foundation as well as with the practical instructions needed to carry out the measurements. At the same time, a high scientific standard is maintained throughout the book, and, in pointing out the mistakes the beginner is liable to make, the author reveals the strength of his critical judgment. His attitude towards the reader, namely, that of a helpful, experienced friend toward a novice, lends a lively note to the text. The book consists of four parts. The first, "A Rheological System," gives an extremely lucid statement of the theoretical basis. The second part, "The System Applied to Laboratory Measurements," describes the instruments, their application, and the correct interpretation of the data. The third and fourth parts, "The Particle—the Basis of Rheological Structure" and "Particle Groups Rheological Structures," outline the correlation of rheological and microscopical observations. The appendix contains among others two brief but delightful chapters advising the laboratory investigator and the laboratory director of the proper course to be followed in their effort to obtain understanding and support of their activities from the executives.

The book is well illustrated and indexed. The literature references do not intend to cover the complete field of rheology but rather to select the useful.

The book was primarily written for the control technologists on the field of printing inks and paints, but will be of value also to the investigators of non-Newtonian liquids generally, which include numerous biochemical systems.

E. I. VALKO, Mountain Lakes, N. J.

**Introduction to Radiochemistry.** By GERHART FRIEDLANDER AND JOSEPH W. KENNEDY. John Wiley, New York; Chapman and Hall, London, 1949. xiv + 412 pp. Price \$5.00.

Recognizing the present lack of precision in usage of the terms radiochemistry, nuclear chemistry, tracer chemistry, and radiation chemistry, the authors define radiochemistry to embrace both nuclear chemistry and tracer chemistry (but not radiation chemistry).

This book is intended as a text of radiochemistry for students of chemistry at the graduate level who have had no special preliminary training in physics. This latter condition has enjoined the authors to present a more or less complete treatment of the facts of nuclear physics. Because this necessary basic information occupies 235 of the total of 292 text pages, the book title might well indicate the book as an introductory text of nuclear radiation physics and radiochemistry.

The basic facts of nuclear radiation physics are presented in logical and intelligible fashion. Most of the chapters are concluded with a series of good illustrative problems, and a list of references to the original literature and other texts of nuclear physics.

The presentation of the chemistry in the last three chapters is disappointingly brief, particularly in the discussion of coprecipitation and adsorption, the tracer method and its pitfalls, and the applications to analytical chemistry.

All in all, however, the text will meet a need and can be recommended for an introductory course in this rapidly developing borderline field.

LEON L. MILLER, Rochester, New York

**An Introduction to Plant Biochemistry.** By CATHERINE CASSELS STEELE. 2nd rev. ed. G. Bell and Sons, London, 1949. viii + 346 pp. Price 22s. 6d.

This book is a second, revised edition of the one which appeared in 1934. Since 1934 a wealth of new material has appeared both in the organic chemistry of plant products and particularly in the study of plant metabolism. These newer findings are in part incorporated into the present edition of Steele's book. In many instances new concepts are grafted onto old with a resultant lack of clarity which will no doubt confuse the student of botany at whom the book is aimed, avowedly to provide him "... with an introductory account of the chemical nature and relationships of the substances elaborated by plants."

The framework of this book is primarily organic chemical, an introductory section, I, on the colloidal state, being followed by sections on II, alcohols, fatty acids, fats and oils; III, aldehydes, ketones, and carbohydrates; IV, plant acids; V, proteins and related compounds; and VI, phenols, aromatic alcohols *etc.*, pigments, alkaloids, and essential oils. Only in the final section (VII, plant metabolism), comprising 74 of the total 319 pages, does the author consider general metabolic processes including photosynthesis, respiration, nitrogen metabolism, and growth. The sections on the various categories of plant products are in many respects the most useful in the book. Each group of substances is introduced by an elementary discussion of the organic chemistry involved, and simple experiments illustrating chemical properties are appended to the discussions throughout the text. The chapters on glycosides and essential oils are particularly good as brief surveys of the nature and distribution of these groups of compounds. Other chapters are less satisfactory. Thus the discussion of cell-wall polysaccharides gives no over-all picture of cell-wall structure and composition and little insight into the complex subject of noncellulosic polysaccharides and polyuronide hemicelluloses. Pectic substances are discussed in less than one page and no clear differentiation between protopectin, pectin, and pectic acid is made. The old concept that pectic substances have to do with cementing cellulose fibers together is again brought forward. The discussion of lignin is similarly brief and uninformative.

The section on plant proteins is almost completely confined to the seed proteins and makes little reference to our extensive knowledge of cytoplasmic proteins and their functions.

In the course of the sections on natural products the author brings forward short sections on biogenesis and function, the latter often amusingly teleological. Thus fat formation "plays an important part in the defense mechanism of the plant against low temperatures." Glycosides "perform a biological function in protecting the plant from raids by animals. . . ." Anthoxanthins play a role in ". . . protecting the plant from over-insolation," although anthocyanins may also act ". . . by converting the absorbed rays into heat (and) actually raise the temperature of the tissue." The discussions on biogenesis are frequently not based on current information and hark back to the earlier edition. Thus a page is devoted to formaldehyde as a progenitor of carbohydrate, uracil is suggested to arise from malic acid and urea, *etc.*

It is in its biochemical aspects particularly that Steele's book leaves much to be desired. The central role of the plant acids in respiration is not stressed and is in fact barely mentioned in the chapter on the plant acids, although it is taken up to some extent in the chapter on respiration. Isocitric acid is not mentioned among the plant acids even in the extensive discussion of the succulents in which isocitric is the principal acid. Of the discussions under Plant Metabolism in section VII, only that on fruit ripening can be considered as enlightening. The chapter on enzymes is vague and relies on much old material, citing for example the plastein work of Borsook and Wasteneys (1925) 'as a case of enzymatic synthesis. The chapter on respiration is not logically organized and conveys no clear picture of the biochemistry of the process. The Krebs cycle (together with carbon dioxide fixation) is discussed in one page, concluding with the remark, "Isocitric acid is the most oxidizable substance in the cycle and experiments indicate that final oxidation to carbon dioxide and water occurs at this point. . . ." Plant growth is taken up in a most desultory manner. Among the few references to recent work is a curious statement to the effect that "Wendt, Kögl and their collaborators" have isolated indoleacetic acid from malt and maize germ oil.

This book is unfortunately full of minor and major errors of fact and interpretation. Among the most glaring are those having to do with enzymatic matters. Thus starch phosphorylase is attributed a role in sucrose synthesis; oxidative deamination is referred to as transamination; hexokinase, phosphorylase, and phosphatase are apparently used almost interchangeably in various sections of the book.

This book does then have its virtues as a brief guide to the plant products. At the same time it leaves much to be desired with respect to logic of organization, and clarity and accuracy in presentation.

JAMES BONNER, Pasadena, Calif.

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